EFFECT OF ENVIRONMENTALLY-DERIVED SEX STEROIDS ON COXSACKIEVIRUS B3 MYOCARDITIS: FOCUS ON VITAMIN D AND BISPHENOL A

by
Katelyn Ann Bruno

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ABSTRACT

Effect of Environmentally-derived Sex Steroids on Coxsackievirus B3 Myocarditis: Focus on Vitamin D and Bisphenol A

Katelyn Ann Bruno

Advisor:

DeLisa Fairweather, PhD

Readers:

Alan Scott, PhD

Kathleen L. Gabrielson, DVM, PhD, ACVP

Winnie Y. Tang, PhD

ABSTRACT

Myocarditis is an inflammatory heart disease that leads to DCM and heart failure. Sex hormones play a vital role in the development of myocarditis with testosterone driving disease in males. In contrast, estrogen mediates cardioprotection in females. Since myocarditis is influenced by sex hormones, it is highly probable that environmental factors like vitamin D and endocrine disruptors like bisphenol A (BPA) and BPS, which interfere with natural hormone signaling, could play a part in the progression of the disease. To our knowledge no one has examined the role of vitamin D receptor or endocrine disruptors like BPA on myocarditis. In order to examine the role of vitamin D on viral myocarditis, we examined vitamin D receptor knockout mice fed a calcium-supplemented diet to prevent rickets. For BPA and BPS experiments, we exposed mice to various doses of endocrine disruptors dissolved in their drinking water for two weeks and then examined their effect on viral myocarditis. We found that vitamin D receptor signaling protects female mice from myocarditis, but increases myocarditis in males. For endocrine disruptor exposure we found that BPA and BPS were each able to increase viral myocarditis by increasing mast cell numbers/ degranulation and T cells in female BALB/c mice. BPA exposure was also found to increase viral myocarditis in male BALB/c mice. In contrast, BPA exposure had no significant effect on myocarditis in female C57BL/6 mice. Surprisingly, housing mice in plastic cages was able to cause mast cell activation in females and increase myocarditis in male BALB/c mice. These data indicate that the traditional cages that investigators use to house their mice may be altering the immune response in a sex-specific manner. Our data show that exposure to endocrine disruptors significantly alter the immune response according to sex and mouse strain.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Abbrev	
%EF	% ejection fraction
%FS	% fractional shortening
-/-	knock out
+/-	heterozygous
+/+	wild type
ΔCt	delta Ct
4E-BP1	E-binding protein-1
AR	androgen receptor
Arg1	arginase-1
BCR	B cell receptor
BL/6	C57BL/6
BPA	bisphenol A
BPF	bisphenol F
BPS	bisphenol S
BW	body weight
Casp1	caspase-1
Ccl	chemokine C-C motif ligand
Con	control
CR	complement receptor
Ct	cycle threshold

CVB	coxsackievirus B
CVD	cardiovascular disease
Cyp	cytochrome P450
Cyp24A1	1,25-dihydroxyvitamin D3 24-hydroxylase
Cyp27B1	25- dihydroxyvitamin D3-1α-hydroxylase
Cyp2R1	25-hydroxylase
Cxcl	C-X-C motif chemokine
DBP	VitD binding protein
DC	dendritic cell
DCM	dilated cardiomyopathy
E2	17β-estradiol
EAM	experimental autoimmune myocarditis
ED	endocrine disruptor
ELISA	enzyme linked immunosorbent assay
EPA	Environmental Protection Agency
ER	estrogen receptor
ERRγ	estrogen-related receptor gamma
FACS	Fluorescence-activated cell sorting
GCM	giant cell myocarditis
GDX	gonadectomy
GPR30	G-protein coupled receptor for estrogen 30
H&E	hematoxylin and eosin
HPRT	hypoxanthine phosphoribosyltransferase 1

IC	immune complex
iC3b	inactivated C3b
IFN	interferon
IL	interleukin
IL-1R	interleukin 1 receptor
ILC	innate lymphoid cell
Inadeq	inadequate
ip	intraperitoneally
IRB	Institutional Review Board
LC-MS/MS	liquid chromatography-mass spectrometry/ mass spectrometry
LM	lymphocytic myocarditis
LOAEL	lowest advese effect level
LV	left ventricular
MC	mast cell
MDSC	myeloid derived suppressor cells
MHz	megahertz
Mild Def	mildly deficient
Mmp	matrix metalloproteinases
MSC	mesenchymal stem cell
MyD88	myeloid differentiation factor 88
NK	natural killer
NKT	natural killer T cell
OVX	ovariectomy

PAMP	pattern associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
P-ERα	phosphorylated- ERα
P-ERβ	phosphorylated - ERβ
PFU	plaque forming units
pi	post infection
PPCM	peripartum cardiomyopathy
qRT-PCR	quantitative real time-polymerase chain reaction
RA	rheumatoid arthritis
RWT	relative wall thickness
RGE	relative gene expression
sc	subcutaneously
SCF	stem cell factor
SEM	standard error of the mean
SLE	systemic lupus erythematosus
sST2	soluble ST2
Te	testosterone
TGF	transforming growth factor
Th	T helper
TIMP-1	tissue inhibitor of metalloproteinases
TLR	Toll-like receptor
TNF	tumor necrosis factor

Treg	regulatory T cells
TRIF	TIR-domain-containing adapter-inducing interferon-β
US	United States
UVB	ultra violet B
VDR	VitD receptor
VDRE	VitD response elements
VitD	vitamin D (1α,25-dihydroxyvitamin D3)
WT	wild type
Ym1	chitinase 313

LIST OF TABLES

Chapter 3

LIST OF FIGURES

FIGURE **PAGE**

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Chapter 8

Chapter 9

Chapter 1

Introduction

Epidemiology of Autoimmune Diseases

Myocarditis, defined as an inflammation of the myocardium, is considered to be an autoimmune disease. Myocarditis can progress to dilated cardiomyopathy (DCM), a leading cause for needing a heart transplant (Cooper 2009A). Autoimmune diseases are acute and chronic inflammatory conditions where memory-specific T and B cells and antibodies are directed against self-tissues and there has been a breakdown of "tolerance" to self (Rose 2006). Autoreactive T and B cells occur frequently in individuals but do not necessarily lead to autoimmune disease. Epidemiology studies have shown that autoimmune disease usually only develops when multiple autoantibodies are directed against a target tissue (Notkins 2004, Fairweather 2008A). Autoimmune diseases have been estimated as the third most common category of chronic disease in the United States (US) after cardiovascular disease (CVD) and cancer, if they are considered as a group (Fairweather 2008A).

Over 80 individual autoimmune diseases or autoimmune syndromes have been identified (Hayter 2012). Autoimmune diseases have been estimated to affect around 5-8% of the population (Jacobson 1997). The study by Jacobson et al was one of the first to examine autoimmune diseases as a group; they included 24 autoimmune diseases in their analysis (Jacobson 1997). A study of 31 autoimmune diseases found that the prevalence of autoimmune disease in Denmark from 1977 to 2001 was 5.3% (Eaton 2007). The prevalence estimates from the Denmark study are considered to significantly underestimate the true prevalence of autoimmune diseases because they are based on hospitalization registry data. Cooper et al updated the prevalence of 29 autoimmune diseases worldwide from 1989 to 2008 and estimated a higher incidence ranging from 7.6% to 9.4% (Cooper, 2009B). A more recent study examining the prevalence of 81 autoimmune diseases found an overall

prevalence of 4.5% (Hayter 2012). Thus, autoimmune diseases are a major cause of morbidity and financial burden to the health systems of Western countries.

Sex differences in epidemiology of autoimmune diseases

One of the earliest epidemiology studies to report sex differences in autoimmune diseases was Beeson in 1994 (Beeson 1994). Beeson reported that out of 40 autoimmune diseases the highest female bias compared to males were for patients with Sjögren's syndrome (19:1), systemic lupus erythematosus (9:1), and Hashimoto's thyroiditis (6:1) (Beeson 1994). Jacobson et al also reported that autoimmune diseases are more common in women. They reported that women were at a 2.7 times greater risk of developing an autoimmune disease compared to men, and estimated that 79.8% of 24 autoimmune diseases occurred in women (Jacobson 1997). Examples of autoimmune diseases that occur more frequently in women than men with more recent prevalence estimates include Hashimoto's thyroiditis (19:1), Sjögren's syndrome (16:1), scleroderma (also called systemic sclerosis) (12:1), systemic lupus erythematosus (7:1), rheumatoid arthritis (3:1), and myositis (2:1) (Zandman-Goddard 2007, Hayter 2012). The top 5 most prevalent autoimmune diseases in men and women according to the epidemiology study by Hayter and Cook are rheumatoid arthritis, Hashimoto's thyroiditis, celiac disease, Graves' disease, and type I diabetes (Hayter 2012). However, the most prevalent autoimmune diseases are not necessarily those with the highest sex bias. Hashimoto's thyroiditis has a high prevalence and sex bias (19:1 women to men), but the other 4 most prevalent autoimmune diseases do not have a high sex bias. Sjögren's syndrome and scleroderma have a high sex bias in women, but a relatively low prevalence compared to the most prevalent autoimmune diseases.

In contrast, with the exception of type I diabetes and Crohn's disease, most autoimmune diseases that are more common in men occur rarely (Hayter 2012). Autoimmune diseases that occur more frequently in men have a smaller sex bias of only about 2:1 for men compared to women. Inflammatory conditions that occur more frequently in men like cardiovascular diseases, many cancers (i.e., lung, liver, stomach), and the leading autoimmune disease in men, type I diabetes, are leading causes of death in men (Regitz-Zagrosek 2010, Dorak 2012, Fairweather 2012A, Soerjomataram 2012, Vos 2012, Fairweather 2013). In contrast, inflammatory diseases that occur more often in women like autoimmune diseases, allergy and asthma tend to have lower mortality (Fairweather 2008A, Lim 2012, Townsend 2012).

Sex differences in the epidemiology of myocarditis and DCM

Myocarditis and DCM also occur more frequently in men, but they are left out of most epidemiology studies of autoimmune diseases. In 1949, Ludden and Edwards from the Mayo Clinic reported a 2.5:1 ratio of myocarditis in men to women following a poliomyelitis outbreak that occurred in Minnesota in 1946 (Ludden 1949). A study of myocarditis patients in 1968 reported that 72% of cases occurred in men compared to women (Sainani 1968). Similarly, in 1980 Woodruff conducted a survey of 164 cases of viral myocarditis reported in reviews from 1957 to 1973 and found that 67% of patients were male (Woodruff 1980). Recent trials and registries of myocarditis report a female to male ratio of around 1:1.7 (Mason 1995, Magnani 2006, Caforio 2007), while DCM studies report a female to male ratio of around 1:1.5 (Bagger 1984, Gillum 1986, Coughlin 1993). In 1989, a population-based study found the age-adjusted female to male ratio for both incidence and prevalence of

idiopathic DCM to be 1:3 (Haddad 2008). There are no recent epidemiology studies of the prevalence or incidence of myocarditis. However, McNamara et al found that myocardial recovery was significantly better for women than men with myocarditis/ acute DCM in the Intervention in Myocarditis and Acute Cardiomyopathy (IMAC)-2 Study (McNamara 2011). Transplant-free survival was also significantly better in women than men in that study. The increased incidence of men developing idiopathic DCM compared to women was not explained by socioeconomic factors, alcohol intake or other variables (Dec 1994, Aguero 2008). Thus, the incidence of myocarditis appears to be similar to other male-dominant autoimmune diseases like type I diabetes, with an approximately 2:1 ratio of men to women.

Pathology of Myocarditis and DCM

Pathology of Myocarditis

Myocarditis was listed in a 2012 *Lancet* report as the 32nd cause of death globally (Lozano 2012). Myocarditis can occur at any age in men and women, but most cases in the US occur during peak reproductive years so that myocarditis is the leading cause of sudden death (aside from accidents) in the US in adults under the age of 40 (Drory 1991, Huber 1997). From 4-20% of sudden cardiovascular deaths among young adults, the military, and athletes are believed to be due to myocarditis (Gupta 2008). In some individuals, myocarditis can progress to DCM and heart failure (Cooper 2009A, Wexler 2009, Roger 2011).

Myocarditis is defined according to "the Dallas criteria" as an inflammatory infiltrate of the myocardium with or without necrosis (Aretz 1987). Myocarditis can develop in response to infections (i.e., viruses, bacteria, and parasites), toxins, chemicals, allergic reactions and/or drugs, and is considered an autoimmune heart disease (Gauntt 2003, Li

2006, Cooper 2009A, Guglin 2012, Marchant 2012, Fairweather 2013, Reddy 2013). The main cause of myocarditis worldwide is believed to be viral infections, which in the US population is believed to be mainly due to coxsackieviruses (CVB). However, the true incidence and prevalence of myocarditis are unknown due to the unavailability of safe and accurate noninvasive diagnostic tests (Cooper 2009A, Schultheiss 2011A, Schultheiss 2011B). Myocarditis has been estimated to be the cause of death in less than 1% of autopsy cases (Kyto 2007), but a cause of unexpected death in young adults around 17% of the time (Doolan 2004). Although most cases of suspected myocarditis are not linked to a specific cause (Schultheiss 2011A, Schultheiss 2011B), viral infections like CVB are the most commonly identified cause of myocarditis in developed countries (Gupta 2008, Cooper 2009A). CVB is nearly 100% prevalent in the population worldwide where individuals are usually infected as an infant/ child and re-infected each year. CVB is unique in that it has no known species barrier, and has no known sex difference in infectivity in humans or mice (Frisancho-Kiss 2007, Coronado 2012, Fairweather 2012A, Fairweather 2012B). Antiviral treatments with interferon (IFN)-β have been shown to reduce myocarditis and DCM in animal models and patients (Kuhl 2003, Wang 2007), further suggesting that viral infections are an important cause of myocarditis/ DCM cases in patients.

Infections are also believed to induce autoimmune disease (Huber 2006, Fairweather 2007, Ellis 2010, Root-Bernstein 2015). There are a number of theories on how viruses can cause autoimmune diseases including molecular mimicry, epitope spread, an adjuvant effect, or simply a direct effect of infection (Root-Bernstein 2014). CVB3 induces autoimmune myocarditis that progresses to DCM in susceptible strains of mice like A/J and BALB/c that closely resembles disease progression in people (Fairweather 2001, Gaunt 2003, Fairweather

2012A, Fairweather 2012B, Fairweather 2013, Myers 2013). Black background mice like C57BL/6 develop severe acute CVB3 myocarditis, but are resistant against developing DCM (Fairweather 2001, Abston 2012A). CVB3 myocarditis in C57BL/6 mice is more severe than disease in BALB/c mice during acute myocarditis, and appears to be similar to fulminant forms of myocarditis in the patient population. T helper (Th)1 /IFN responses increase acute myocarditis in male mice (Fairweather 2004A, Fairweather 2005, Frisancho-Kiss 2006, Fairweather 2012B). However, the same elevated Th1 response that increases acute myocarditis prevents progression to DCM by preventing remodeling in the heart (Abston 2012A). A recent study of myocarditis/ acute DCM patients in the US found that more men than women developed DCM following myocarditis (McNamara 2011). More male BALB/c mice progress to DCM following CVB3 myocarditis compared to females (Fairweather 2001, Frisancho-Kiss 2007, Coronado 2012, Fairweather 2013), similar to the sex difference that exists for DCM in people.

Similar to findings in clinical biopsies of myocarditis patients (Cooper 2009A, Fairweather 2014), the primary infiltrate in mouse models of myocarditis consists of macrophages and neutrophils with lower levels of T cells, B cells, mast cells and dendritic cells (DC) (Huber 1983, Frisancho-Kiss 2007, Cihakova 2008). Natural killer cells, CD8 T cells and γδT cells, important in antiviral defense, are present in the heart during the early cellular response in viral animal models of myocarditis (Fairweather 2001, Huber 2002). Acute myocarditis in mice is characterized by a predominantly Th1 and Th17 response (Huber 2002, Baldeviano 2010, Yuan 2010, Noutsias 2011, Fairweather 2012B). However, only mice that have higher levels of mast cells, such as BALB/c and A/J strains, and respond to infection with a Th2 response progress to develop DCM (Afanasyeva 2001, Fairweather

2004A, Fairweather 2004B, Frisancho-Kiss 2006, Fairweather 2008B, Abston 2012A, Abston 2012B). In many clinical cases, and in the CVB3 animal model, the pericardium as well as the myocardium can be affected, which is referred to as perimyocarditis or myopericarditis (Abston 2012B, Imazio 2013). Mast cell (MC) degranulation occurs most frequently along the pericardium (Fairweather 2004A), and when this occurs mice and humans are at an increased risk of progression to DCM and heart failure (Fairweather 2004, Fairweather 2008, Abston 2012B, Mina 2013).

Pathology of Dilated Cardiomyopathy

DCM is defined as a heterogeneous group of diseases of the myocardium associated with mechanical and/or electrical dysfunction that exhibit inappropriate ventricular hypertrophy or dilation and are due to a variety of causes (Maron 2006). DCM is considered the most common form of cardiomyopathy (Cooper 2009A). Survival of patients with DCM is only 50% five years after diagnosis and is a leading cause for needing a heart transplant (Maron 2006). The cause of most cases of DCM is unknown, except for familial/genetic cases, and so the disease is frequently termed “idiopathic” DCM. Regardless of the cause of DCM, the pathogenesis of disease follows a similar progression. For DCM to develop the heart most typically must undergo remodeling and fibrosis.

Experimental Mouse Models of Myocarditis and DCM

Three main mouse models of myocarditis exist: 1) the classic CVB3 model that uses tissue culture-derived or purified virus (virus-only), 2) an experimental autoimmune myocarditis (EAM) model induced by adjuvant and cardiac myosin or cardiac myosin

peptides, and 3) a hybrid-CVB3 model that is induced using heart-passaged CVB3 and damaged heart proteins (Cihakova 2004, reviewed in Fairweather 2012B, Myers 2013). Each model contributes uniquely to our understanding of the pathogenesis of disease.

CVB3-only model

Tissue culture-derived CVB3 is the most commonly used method to induce viral myocarditis. Mice are infected intraperitoneally (ip) with purified virus or RNA from various CVB3 strains (i.e., Woodruff, H3, Nancy) after propagation in HeLa cells. This method was originally described in 1974 by Woodruff et al (Woodruff 1974). In this model, viral replication peaks in the heart around day 5-7 post infection (pi) (10^7-10^9 plaque forming units/PFU/g heart) but only causes low levels of inflammation in the heart (5-10% of heart tissue inflamed) followed by rapid heart failure (Gaunt 2003). A problem with using this model is that only about 30% of the mice survive to day 7 pi (**Table 1**) (Gaunt 2003). Myocarditis using this model is more severe in male compared to female mice (Huber 1981, Huber 2005, Li 2009). An innate immune cell type called γδT cells is critical for disease induction in this model (Huber 2002, Liu 2011). More severe myocarditis is associated with a Th1 response- particularly in male mice (Huber 2002). The presence of autoreactive T and B cells in this model indicates that autoimmunity is involved in disease pathogenesis (Huber 2006).

EAM Model

EAM is induced using adjuvants (i.e., inactivated *Mycobacterium* and/or Pertussis toxin) and cardiac myosin peptides, which are typically injected on day 0 and 7 (Cihakova

2004). Peak myocarditis occurs at day 21 (**Table 1**) (Barin 2011, Rose 2011). In EAM, myocarditis consists mainly of macrophages and neutrophils with lower amounts of T and B cells, mast cells and dendritic cells (Cihakova 2008, Barin 2011). Only Th2 responding strains like BALB/c and A/J develop EAM and progress to DCM (Afanasyeva 2001). DCM develops around day 42 post inoculation, when fibrosis and necrosis is observed (Afanasyeva 2001, Afanasyeva 2004). A Th1 immune response has been shown to prevent acute and chronic EAM (Afanasyeva 2004, Barin 2010), while a Th17 response increases chronic fibrosis and DCM (Baldeviano 2010).

Hybrid-CVB3 model

The so-called "hybrid" model of CVB3 is the model used in this thesis. Myocarditis is induced by propagating the virus in culture in Vero cells (monkey kidney cells) and then infecting mice and collecting the heart, homogenizing and then using the homogenate, which contains infectious virus and damaged heart tissue, to infect mice (Fairweather 2007). The injection of mice with damaged self/ heart tissue as well as infectious virus is why this model has been called a "hybrid" or viral-autoimmune model. Mice develop autoantibodies against cardiac myosin and immune complexes (ICs) deposit in the heart (Fairweather 2001, Fairweather 2006), indicating the importance of the autoimmune component in the pathogenesis of disease in this model. All mouse strains that have been tested so far including BALB/c, A/J, C57BL/6, and mixed backgrounds develop acute myocarditis that peaks at day 10 pi. Similar to EAM, acute myocarditis consists mainly of macrophages and neutrophils with lower numbers of T and B cells, mast cells, and dendritic cells (**Table 1**) (Frisancho-Kiss 2007, Cihakova 2008, Barin 2011). Viral replication peaks around day 7 pi and is

cleared from the heart by day 14 pi (Fairweather 2001, Fairweather 2007). In contrast to the virus-only model, all wild type (WT) mice survive acute myocarditis (Fairweather 2001). Again similar to EAM, only susceptible Th2 responding strains of mice like BALB/c and A/J develop chronic myocarditis and DCM (Fairweather 2004A, Fairweather 2004B, Fairweather 2007, Abston 2012A, Abston 2012B). DCM develops in this model by day 35 pi (Fairweather 2004A). Cardiac fibrosis and necrosis are only found in the heart during the chronic myocarditis/ DCM stage, but not during acute myocarditis. This hybrid-CVB3 model more closely resembles EAM than CVB3-only models (**Table 1**) (Afanasyeva 2004, Fairweather 2004A, Cihakova 2008). Myocarditis and DCM are more severe in male BALB/c mice (Frisancho-Kiss 2007, Onyimba 2012, Coronado 2012), similar to myocarditis patients (McNamara 2011). Increased myocarditis in male mice is a common feature of all three myocarditis mouse models (**Table 1**).

Table 1. Summary of animal models of myocarditis/DCM[a,b]

	CVB3-only model	Hybrid-CVB3 model	EAM
Survival	20-30% by day 7 pi	100% to day 90 pi	100% to day 90 pi
Viral replication d7 pi	10^7-10^9 PFU/g heart	10^5 PFU/g heart	0
Acute myocarditis	Peak @ day 7 pi	Peak @ day 10 pi	Peak @ day 21
Severity of myocarditis	5-10% inflammation	30-60% inflammation	30-60% inflammation
Key cell mediators	γδT & $CD8^+$ T cells	Macrophages	Macrophages
DCM	Few survive	Yes	Yes
Sex differences	Males > females	Males > females	Males > females

[a] CVB3, coxsackievirus B3; d, day; EAM, experimental autoimmune myocarditis; pi, post infection; PFU, plaque forming units; DCM, dilated cardiomyopathy
[b] from Fairweather 2012B

Innate Immune Response in CVB3-induced Myocarditis

The innate immune response allows discrimination of self from non-self and launches an immune response to pathogens or other agents. Innate immunity determines the type of adaptive immune response that will develop based on the type of infectious agent that initiates the immune response. The innate immune response is particularly important in the pathogenesis of myocarditis. Innate immune mediators like macrophages, mast cells, the inflammasome, interleukin (IL)-33, and complement have been found to increase myocarditis following CVB3 infection in our hybrid model of myocarditis (Fairweather 2003, Fairweather 2004B, Fairweather 2006, Abston 2012B, Coronado 2012).

Complement

Complement and complement receptors (CRs) are central to the innate immune response by promoting phagocytosis, activating the adaptive response, rapidly destroying invading microorganisms, and by clearing ICs (Morgan 1999, Kemper 2007, Dunkelberger 2009, Arbore 2016). Deficiencies in complement and CRs are known to increase susceptibility to certain infections like group B *Streptococcus* and to increase IC-mediated autoimmune diseases like systemic lupus erythematosus (Morgan 1999). Activation of the complement cascade by infection, for example, generates complement component C3, which is cleaved by C3 convertase to produce C3a and C3b. C3a binds C3aR1 on mast cells (**Fig 1**). C3b binds CR1 on mast cells and macrophages, which inhibits C3 convertase to reduce inflammation (Fairweather 2006). C3b is further processed to become inactivated C3b (iC3b), which binds CR3 (a heterodimer of CD11b and CD18) on mast cells and macrophages (**Fig 1**). CD11b is also called Mac1 or Itgam. Because CR2 (CD21) only binds

inactive C3 (i.e., C3d), it does not regulate complement activation. Instead CR2 is mainly a part of the B cell receptor (BCR) complex that is needed to activate B cells (Roosendaal 2007, Jacobson 2008). CD19, which forms part of the BCR complex, is an important regulator of B cells (Fujimoto 2007*).*

In contrast, CR1 (CD35) binds C4b and C3b, and inhibits C3 and C5 convertase activity involved in the classical, alternative, and lectin complement pathways. CR1 is also important for clearing ICs, which protects the host from complement-mediated tissue damage (Fairweather 2006). While CR1 and CR2 are encoded on separate genes in man, in the mouse they are derived from the same gene, *Cr2*, so that mice deficient in one receptor are also deficient in the other (Kurtz 1990). During acute CVB3 myocarditis, CR1/2 is primarily expressed on mast cells, $GR1^{+}F4/80^{+}$ alternatively activated M2 or myeloid-derived suppressor-type macrophages (MDSCs), and $GR1^{hi}$ neutrophils (**Fig 1**) (Fairweather 2006). CR1/2 deficient mice have significantly increased CVB3 myocarditis with elevated CD11b+ immune cells (i.e., macrophages and mast cells) and IL-1β (Fairweather 2006). Disease is so severe in these mice that they rapidly develop myocardial and pericardial fibrosis and progress quickly to DCM and heart failure (Fairweather 2006), indicating the critical role for complement in driving CVB3 myocarditis. Complement is also important in driving EAM (Kaya 2001).

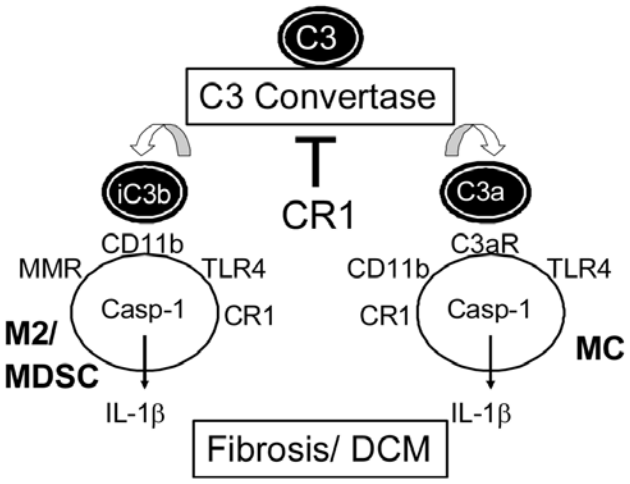


Figure 1. Model of CR1 regulation of macrophages, mast cells, fibrosis and DCM in males. Following infection with CVB3, C3 is converted to C3b and C3a by C3 convertase. C3b binds CR1 and iC3b binds CD11b/CD18 (CR3) on macrophages (M2/MDSC) and mast cells (MC) while Toll-like receptor (TLR)4 and caspase-1 (casp-1) generate active interleukin (IL)-1β, a potent profibrotic cytokine. C3a binds C3aR on mast cells, which also express TLR4, caspase-1 and IL-1β. CR1 expressed on macrophages and mast cells inhibits C3 convertase in a negative feedback loop. We observe increased numbers of $CD11b^{+}$, M2/MDSC and mast cells and IL-1β levels in the heart of CR1/2 deficient mice that is associated with increased fibrosis and accelerated DCM (Fairweather 2006). Since CR2 does not possess complement regulatory functions, we hypothesize that CR1 is responsible for protecting against the development of fibrosis and DCM in CR1/2 deficient male mice by inhibiting IL-1β production from macrophages and mast cells (Fairweather 2006).

TLR4 and the inflammasome

Toll-like receptors (TLRs) are part of a large group of receptors called pattern recognition receptor (PRRs) that play an important role in the innate immune system by recognizing and binding pathogen-associated molecular patterns (PAMPs) on infectious organisms allowing a pathogen (or toxin)-specific immune response (Janeway 2002). In this way TLRs allow recognition of self vs. foreign antigens. Thirteen TLRs have been described, with only 10 of these expressed in humans. TLR4 is the most highly expressed TLR in the heart, followed by TLR2 and TLR3 (Nishimura 2005). TLR4 is expressed on cardiac myocytes as well as DCs, mast cells and macrophages (**Fig 1**). TLR4 is unique because it responds to many different agents including lipopolysaccharide from bacteria, respiratory syncytial virus, and damaged self- including heat shock proteins and hylauronan. Recently, complement C3a has been found to activate the inflammasome leading to increased IL-1β expression from macrophages and dendritic cells (Laudisi 2013, Agari 2013, Arbore and Kemper 2016). TLR4 is expressed on the cell surface usually in a complex that includes CD14, LPS binding protein, and MD2 (Krishnan 2007). TLR4 is also unique among TLRs because it can signal via the adaptor protein myeloid differentiation factor 88 (MyD88) or TIR-domain-containing adapter-inducing interferon-β (TRIF) (Abston 2012A).

TLR4 signaling is required to generate pro-IL-1β and pro-IL-18 that is then cleaved by caspase-1 (casp-1) to release active IL-1β and IL-18 from cells. TLR4 expression is upregulated on innate immune cells like mast cells and macrophages from the spleen and peritoneum within hours of infection with heart-passaged CVB3 (Frisancho-Kiss 2006, Frisancho-Kiss 2007). However, it is not known if this is due to direct binding or indirect activation via complement or damaged heart components, for example. Elevated TLR4

expression on mast cells and macrophages is a feature of the male-dominant immune response in the spleen and heart following CVB3 infection (Frisancho-Kiss 2006, Frisancho-Kiss 2007, Coronado 2012). TLR4 deficient male mice develop significantly less acute CVB3 myocarditis with decreased levels of IL-1β and IL-18 in the heart after infection (Fairweather 2003), indicating an important role for the TLR4 signaling pathway in increasing myocarditis in males. Additionally, cardiac myosin has been found to signal via TLR2 and TLR8 in myocarditis patients (Zhang 2009), indicating that TLR activation is also important in driving autoimmune mechanisms during myocarditis. IL-1β is both strongly proinflammatory and profibrotic and has been shown to increase expression of the remodeling gene Serpin A3n during acute CVB3 myocarditis (Coronado 2012). Serpin A3n is also known as α-chymotrypsin, an enzyme released from mast cells that strongly drives tissue remodeling. Testosterone increases both IL-1β and Serpin A3n levels in the heart during acute CVB3 myocarditis (Coronado 2012). IL-1β further elevates Serpin A3n levels, driving cardiac remodeling and fibrosis by reducing matrix metalloproteinase (Mmp)3 and Mmp9 levels in the heart (Coronado 2012).

IL-33 and ST2

IL-33, a member of the IL-1 receptor (IL1R) family that includes TLR4 and the IL1R, is able to increase Th2 responses by signaling through its receptor ST2 located on mast cells, macrophages, Th2 cells and other immune cell types (Liew 2010, De la Fuente 2015, Saluja 2015, Schwartz 2016). Immune cells, most notably mast cells and neutrophils, release proteases that cleave active IL-33 into multiple forms that are 10-30 times more potent (De la Fuente 2015, Saluja 2015, Schwartz 2016). Innate lymphoid cells (ILCs) are the main cell

type that responds to IL-33 and expresses high levels of ST2 ligand (ST2L) leading to increases in cytokine production that influence disease progression such as lung eosinophilia (Schwartz 2016). Soluble (s)ST2 is a serum biomarker predicting progression to heart failure in CVD patients (Shaw 2010). Recombinant (r)IL-33 has been found to reduce inflammation and fibrosis in heart failure animal models by inducing a protective, anti-inflammatory Th2-type immune response (Liew 2010). IL-33 has also been found to reduce left ventricular hypertrophy and fibrosis resulting in improved left ventricular function and survival in animal models of pressure overload and atherosclerosis (Sanada 2007, Liew 2010).

IL-33 is localized to the nucleus of fibroblasts, in particular, and has been termed an "alarmin" because it is released following tissue damage that occurs with cardiac damage or infection (Haraldson 2009, Liew 2010). IL-33 is part of the TLR/IL1R family and binds a receptor complex made up of ST2 and the IL-1R accessory protein, which recruits MyD88 resulting in activation of NF-κB and other transcription factors (Liew 2010). In its soluble form sST2 is believed to function as a decoy receptor for IL-33, reducing IL-33 levels. In patients with acute myocardial infarction and heart failure, elevated serum sST2 is associated with an increased risk of mortality or future heart failure (Shaw 2010).

Paradoxically, IL-33 has been found to increase inflammation and fibrosis in a number of autoimmune diseases in patients and animal models by increasing/ activating mast cells (Liew 2010, Oboki 2010, Saluja 2015). sST2 and/or IL-33 were increased in the sera of patients with rheumatoid arthritis (Fraser 2006, De la Fuente 2015), Crohn's disease (Pastorelli 2010, De la Fuente 2015, Schwartz 2016), ulcerative colitis (Pastorelli 2010, De la Fuente 2015, Schwartz 2016), systemic sclerosis (Manetti 2010), and systemic lupus erythematosus (Mok 2010). Similarly, IL-33 was found to increase experimental autoimmune

encephalomyelitis, an animal model of multiple sclerosis, and collagen-induced arthritis in mice by activating mast cells (Xu 2008, Liew 2010, Oboki 2010). Recombinant IL-33 given to BALB/c male mice was found to significantly increase CVB3 myocarditis and heart failure by inducing pericarditis and increasing IL-1β, IL-6 and IL-4 levels in the heart (Abston 2012B). Thus, a relationship appears to exist between TLR4 and the inflammasome and IL-33/ST2 signaling during CVB3 myocarditis.

Adaptive Immune Response in CVB3-induced Myocarditis

The adaptive immune response is directed by innate immunity to protect the host from infections, toxicants, and other agents/ events that result in damaged self. The hallmark of the adaptive immune response is to provide long-lasting protection to specific foreign (or damaged self) peptides (Doan 2008). Part of the immune strategy to eliminate pathogens and heal damaged tissues involves the release of cytokines. There are three main cytokine phenotypes that are produced in response to pathogens that are effective at eliminating infections, but also characterize diseases like asthma/allergies (i.e., Th2), CVDs (i.e., Th1), and autoimmune diseases (i.e., Th1/Th17) (Fairweather 2004C). CD4+ Th cells can become Th1, Th2 and/or Th17-type immune cells that characteristically release IFNγ, IL-4, and IL-17, respectively, as well as other cytokines (Zhu 2010). This skewing of the immune response down key pathways affects, to some degree, all of the immune cells involved in the adaptive response like macrophages, for example, which can be termed "classically" activated M1 macrophages when their phenotype is changed by an IFN/Th1-type environment or "alternatively" activated M2 macrophages when they are altered by an IL-4/Th2-type immune environment (Siracusa 2008, Fairweather 2009). Markers that are often

used in mice to identify M1 vs. M2 macrophages by qRT-PCR include arginase-1 (Arg-1) and chitinase 313 (Ym1) for M2 macrophages and the Th1-associated chemokines C-X-C motif chemokine (Cxcl)9 and Cxcl10 for M1 macrophages (Siracusa 2008, Fairweather 2009, Abston 2012A).

Role of Th1-type immune responses in CVB3 myocarditis

Many viral infections including CVB3 require IFNs and a Th1-type immune response for effective clearance. In CVB3 models of myocarditis that use tissue-culture derived virus without damaged heart tissue (virus-only models), the inflammatory infiltrate usually closely correlates to the level of viral replication in the heart (**Table 2**) (Huber 2002, Gaunt 2003, Huber 2005, Li 2009, Riad 2011, Yajima 2011, Fairweather 2012B). In contrast, in the hybrid model of CVB3 myocarditis used in this thesis, viral replication levels do not correlate with increased myocarditis unless IFN/Th1 pathways have been disrupted (**Table 2**) (Fairweather 2004A, Fairweather 2005 Frisancho-Kiss 2007, Abston 2012A, Abston 2013).

In 2011 Wiltshire et al compared Th2-responding mouse strains like A/J to Th1-responding mouse strains like C57BL/6 and found that all mouse strains examined upregulated IFN-related genes during myocarditis, and that several of these genes determined susceptibility to infection (e.g., *Fpgt*, *H28*, *Tnni3k*) (Wiltshire 2011). Production of innate IFNβ is known to protect against viral myocarditis in animal models and myocarditis patients (Kuhl 2003, Wang 2007). Protection against viral infection and myocarditis via IFNβ has been found to be mediated by the transcription factor TRIF, which is downstream of TLR3 and TLR4 signaling (Burke 2011, Riad 2011, Xu 2011, Abston 2012A). It has been shown that CVB3 itself tries to limit host antiviral defenses (i.e., IFNβ) by inhibiting mRNA

translation by activating the translational suppressor eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) (Burke 2011). IFNα administration to mice has also been found to decrease CVB3 myocarditis by reducing viral replication in the heart (**Table 2**) (Yue 2011A, Yue 2011B). Yue et al found that inhibiting the IFN-induced chemokines Cxcl10 or chemokine C-C motif ligand (Ccl)2, also called monocyte chemotactive protein-1 (MCP-1), reduced myocarditis by decreasing Th1/ IFNγ responses, but did not alter viral replication in the heart (Yue 2011A, Yue 2011B). These studies suggest that IFNs like IFNα/β may be more important in preventing viral replication in CVB3 myocarditis mouse models, while IFNγ increases inflammation. Although IFNγ increases myocarditis severity, this does not necessarily lead to a "bad" outcome for the heart unless it is too severe (as occurs in fulminant myocarditis cases). For example, mesenchymal stem cells (MSCs) have been found to reduce CVB3-induced myocarditis by increasing IFNγ levels (Miteva 2011, Van Linthout 2011).

Although TLR3 and TRIF deficient mice develop increased myocarditis with elevated cardiac viral replication, only TRIF deficient mice progress to chronic myocarditis/ DCM (Abston 2012A, Abston 2013). TRIF was found to drive the IFNβ response in the heart during CVB3 myocarditis, while TLR3 drove the IFNγ response (Abston 2012A, Abston 2013). TLR3 deficient mice had a significant increase in a IL-4-associated Th2-response while TRIF deficient mice had an increased IL-33-associated Th2-driven response (Abston 2012A, Abston 2013). The IL-4 type immune response in TLR3 deficient mice had only a mild effect on increasing cardiac dysfunction leading to chronic myocarditis and DCM, while the increased IL-33 response in TRIF deficient mice caused severe eosinophilic perimyocarditis, DCM, and early heart failure. Thus, Th2 cytokines can drive the

development of DCM following CVB3 myocarditis by promoting cardiac remodeling and fibrosis.

Role of Th2-type immune responses in CVB3 myocarditis

Although Th1-type immune responses are known to decrease viral replication and prevent progression to chronic myocarditis/ DCM by reducing remodeling, they also increase acute inflammation in the heart (Huber 2002, Fairweather 2004A, Huber 2005, Fairweather 2005, Li 2009). Th2-associated IL-10 and T regulatory cells (Treg) have been found to reduce CVB3-induced myocarditis (**Table 2**) (Frisancho-Kiss 2007, Liu 2011). Administration of cardiac myosin peptide to mice with CVB3 myocarditis reduced cardiac inflammation by increasing IL-10 levels and Treg numbers (Fousteri 2011). Administration of recombinant galectin-9 to mice was found to inhibit acute CVB3 myocarditis by increasing IL-4/ Th2 cells, $GR1^{+}CD11b^{+}$ macrophages and IL-10 in the heart (Lu 2011). γδT cells that produce IFNγ were found to significantly increase myocarditis in "CVB3-only" models of myocarditis by decreasing Treg numbers (Liu 2011). Mast cells are frequently associated with Th2-type immune responses. Histamine receptor-1, which is one of many receptors that induce mast cell activation, was shown to significantly reduce CVB3 myocarditis by decreasing γδT cells and Th1 responses in the heart (Case 2012). Thus, regulatory cells associated with Th2-type immune responses, like IL-10 and Treg, decrease acute CVB3 myocarditis. In contrast, Th2-associated immune responses that activate mast cells, TLR4-TRIF-inflammasome, and M2 macrophages that express TLR4 and release IL-1β are able to increase remodeling in the heart (i.e., Mmps, Serpin A3n) and promote

progression from myocarditis to DCM and heart failure (**Table 2**) (Abston 2012A, Abston 2012B, Coronado 2012).

Role of Th17-type immune responses in CVB3 myocarditis

Elevated IL-17 (i.e., IL-17A) during the acute phase of myocarditis in the EAM model has been shown to increase chronic fibrosis and DCM (**Table 2**) (Baldeviano 2010). Yuan et al showed that IL-17 levels increase in the circulation and heart during the peak of CVB3 myocarditis (day 7-10 pi) (Yuan 2010A, Yuan 2010B). IL-23 and STAT3, which are both important in IL-17 /Th17 immune responses, as well as IL-17 were increased in the heart during acute and chronic CVB3 myocarditis (Yang 2011). Researchers found that Th17-producing cells were increased in the spleen during CVB3 myocarditis compared to controls (Qing 2011, Xie 2011). Blocking IL-17 using neutralizing antibodies in the CVB3-only model was found to improve survival, reduce acute myocarditis, reduce viral replication in the heart, and to increase Treg cells (Fan 2011, Xie 2011A, Xie 2011B). However, CVB3 myocarditis in the hybrid model was not significantly altered in IL-12p40 (IL-23) deficient mice (Fairweather 2012B). IL-23 is a cytokine that is important in promoting IL-17/ Th17-mediated immune responses. Yet co-exposure of mice for 2 weeks with low-dose inorganic mercury prior to infection with CVB3 in the hybrid CVB3 myocarditis model did not significantly increase acute myocarditis, but was found to increase IL-17 levels in the heart which was associated with increased fibrosis and DCM (Nyland 2012). This finding is similar to the role for IL-17 in promoting remodeling and DCM found in the EAM model (Baldeviano 2010).

Table 2. Role of Th immune responses in animal models of myocarditis[a,b]

Cytokine	CVB3-only model	Hybrid-CVB3 model	EAM
IFN-α (Th1)	Inhibits viral replication	ND	ND
IFN-β (Th1)	Inhibits viral replication	Inhibits viral replication Prevents DCM	ND
IFN-γ (Th1)	Inhibits viral replication	Inhibits viral replication Prevents DCM	Prevents DCM
IL-4 (Th2)	Prevents myocarditis	Increases DCM	Increases DCM
IL-33 (Th2)	ND	Increases DCM	ND
IL-23 (p40) (Th17)	Increases myocarditis	No effect on myocarditis	Increases EAM
IL-17 (Th17)	Increases myocarditis	ND	No effect on EAM Increases DCM

[a] Th, T helper; CVB3, coxsackievirus B3; EAM, experimental autoimmune myocarditis; IFN, interferon; IL, interleukin; DCM, dilated cardiomyopathy; ND, no data
[b] from Fairweather 2012B

Sex Differences in the Immune Response During Myocarditis/ DCM

Sex hormone effects on the immune system

Estrogens, testosterone, progesterone, and vitamin D are the major sex hormones that have been studied for their effects on the immune response (Wilder 1995, Lahita 2010). Sex hormones bind to hormone receptors within the cytoplasm of the cell, but can also bind to receptors on the surface of immune cells to mediate rapid responses (Buskiewicz 2016). Sex hormone receptors alter gene expression in cells via androgen and estrogen response elements, which can turn on or off 100's to 1000's of genes at a time (Hammes 2011). These genomic effects are the reason why sex hormones are able to have such large effects on immune function.

Most immune cells express multiple sex hormone receptors that are involved in driving immune responses in sex-specific directions following antigen stimulation. Sex steroid hormone receptors like estrogen receptor (ER)α, ERβ, estrogen-related receptor (ERR)γ and the androgen receptor (AR) are expressed on and within many immune cell populations including mast cells, macrophages, dendritic cells, natural killer cells, T cells and B cells (Gilliver 2010, Buskiewicz 2016). Human and mouse monocyte/ macrophages express ERα, ERβ and the AR (Gilliver 2010, Villablanca 2010). ARs have been found to be expressed more on/in circulating macrophages in men than women (McCrohon 2010). Thus, it is possible that the ratio of sex hormone receptors on any given cell is the key to understanding their effect on immune cell function.

Women respond to infection, vaccination, and trauma with increased antibody production (Styrt 1991, Lang 2004, Cook 2008, Flanagan 2011). Although increased antibody levels protect women from infections, they also increase the risk of developing

autoimmune diseases, particularly female-dominant rheumatic diseases (Beeson 1994, Whitacre 2001, Gharee-Kermani 2005, Gleicher 2007, Zandman-Goddard 2007, Fairweather 2008A, Dube 2009, Pennell 2012). Women may be at an increased risk of developing an autoimmune disease because estrogen activates B cells resulting in increased levels of antibodies and autoantibodies (Straub 2007, Cook 2008, Fairweather 2008A, Lahita 2010, Rubtsov 2010, Fairweather 2012A). In contrast, testosterone decreases B cell maturation, reduces B cell synthesis of antibody, and suppresses autoantibody production in humans.

Sex hormones are able to skew immune responses into characteristic Th1, Th2 and/or Th17 directions because of their ability to influence antigen presenting cells like dendritic cells, mast cells, and monocytes. Sex hormone receptors on T and B cells further drive sex differences in the immune response. Importantly, depending on the context estrogen can either increase Th1/ Th17 responses or increase Th2 responses with very different outcomes on pathology (Fairweather 2012, Papenfuss 2011, Rubtsov 2010, Straub 2007). In cell culture and animal models estrogen has been shown to induce differentiation of dendritic cells, stimulate T cell proliferation, and drive T cells to a Th1 and/or Th17 type immune response by activating the transcription factor NFκB (Straub 2007, Carrers 2010, Khan 2010, Wang 2010, Wahren-Herlenius 2013). However, in other studies estrogen was found to increase Th2 responses, Treg, IL-4-driven alternatively activated M2 macrophages, and the regulatory cytokines IL-4, IL-10, and TGFβ (Polanczyk 2004, Polanczyk 2005, Frisancho-Kiss 2007, Straub 2007, Fairweather 2008A, Xia 2009, Finesh 2010, Pulendran 2010, Rubtsov 2010, Papenfuss 2011, Sellner 2011). Estrogen has been found to be able to inhibit components of the innate immune response including TLR4-induced IL-1β, IL-6, tumor necrosis factor-α and NFκB activation from macrophages (Deshpande 1997, Evans 2001, Frisancho-Kiss

2007, Lahita 2010, Temple 2008). Estrogen can also inhibit transcriptionally Th1-type immune responses resulting in an increased Th2-type immune response (Frisancho-Kiss 2007, Fairweather 2008, Lahita 2010, Pulendran 2010, Fairweather 2012A). Far less research has been conducted on the role of testosterone/ androgens on the immune response. In general, androgens have been found to increase Th1 responses although more research into their function is needed (Giltay 2000, Giron-Gonzalez 2000, Loria 2002, Desai 2004, Frisancho-Kiss 2007, Fairweather 2008A, Lahita 2010, Giron-Gonzalez 2011).

One of the difficulties in studying the effect of estrogens and androgens on immune function is that most researchers do not report whether the cells used in culture or animals used in experiments are male or female (Hammes 2011, Saha Roy 2012, Miller 2014). Equally important is that many researchers have not considered how sex could influence the interpretation of their data (Miller 2012). Experiments involving the immune response that do not report the sex of the cell or animal become essentially uninterpretable. Dose is another very important consideration when assessing the influence of sex hormones on immune function. Low doses of estrogen have been found to increase Th1 responses while higher doses increase Th2 responses (Fairweather 2008, Cutolo 2010). Other important considerations include how testosterone is metabolized to estrogen in different organs/tissues (i.e., aromatase levels), and differences in cytochrome P450 (Cyp) enzyme expression in various target organs which also effect sex hormone metabolism (Straub 2007). Contradictory findings in the literature have in part led to the recent requirement by NIH to include both sexes in government funded studies and to interpret the findings according to sex (Blauwet 2007, The Institute of Medicine of the National Academies 2012, Miller 2012, Miller 2014).

Sex hormone effects on the cardiovascular system

ERα, ERβ, and the AR is expressed on cardiac fibroblasts, cardiomyocytes, vascular smooth muscle cells, and vascular endothelial cells in humans and rodents (Regitz-Zagrosek 2008, Vitale 2009). Aromatase, the enzyme that converts androgens to estrogens, is also expressed in the heart cells. Platelets also express ERβ and the AR and respond to sex steroids (Gilliver 2010). Women have been reported to have higher ER expression in their arteries than men (Vitale 2009). ERα has been shown to protect against vascular injury and atherosclerosis (Hammes 2011, Hodgin 2001). 17β-estradiol has been found to prevent cardiac hypertrophy and fibrosis (Regitz-Zagrosek 2008). Estradiol also inhibits reactive oxygen species-induced cardiac damage and apoptosis in cardiac myocytes (Regitz-Zagrosek 2008). These cardioprotective effects of estrogen are believed to be mediated mainly by signaling through cell membrane ERs rather than traditional genomic receptors (Regitz-Zagrosek 2008). In contrast, ERβ signaling regulates arterial tone and blood pressure.

Testosterone levels in cardiac tissues have been reported to be higher in men than women (Deslypere 1985, Melchert 2001). Male rats that underwent gonadectomy had lower heart weights compared to sham operated rats, suggesting that testosterone promotes hypertrophy (Melchert 2001, Scheuer 1987). Additionally, it is known that men develop atherosclerotic plaques earlier and more extensively than women (Vitale 2009, Regitz-Zagrosek 2008, Fairweather 2012A). The fact that CVDs in women rise after menopause further suggests that sex hormones influence heart disease (Fairweather 2012A). Part of the difficulty in assessing the role of testosterone is that aromatase is able to convert testosterone to estradiol, so that it is difficult to distinguish which hormone is mediating the effect. Additionally, testosterone has many beneficial effects, including metabolism of body fat. Fat

deposits have high aromatase activity, which metabolizes testosterone to estradiol (Jones 2010). Several studies have reported an association between low serum testosterone levels and higher CVD and mortality in older men (Jones 2010, Malkin 2010, Yeap 2010). Testosterone supplementation has also been reported to reduce CVD in older men (Jones 2010, Ohlsson 2011, Yeap 2010). However, a trial of testosterone administration to older men was discontinued because of a higher rate of adverse cardiovascular events in the testosterone group compared to the placebo group (Basaria 2010). There are several possible explanations for the contradictory findings. Lower testosterone levels could indicate that the hormone is being consumed (Onyimba 2011). Testosterone is also known to gradually decrease with age. Also proinflammatory cytokines like tumor necrosis factor-α, IL-1β and IL-6 regulate the hypothalamic-pituitary axis resulting in reduced circulating levels of testosterone (Jones 2010, Hales 1992), and so the CVD itself could be driving down testosterone levels.

Sex differences in myocarditis

CVDs like atherosclerosis and myocarditis occur more frequently in men than women, and men are at an increased risk of developing DCM and heart failure (Cocker 2009, McNamara 2011, Fairweather 2012A, Kyto 2013). Huber and colleagues were the first to describe that male BALB/c mice have increased CVB3-induced myocarditis compared to females (Lyden 1987). Myocarditis is also increased in male BALB/c mice in the hybrid CVB3 model (Frisancho-Kiss 2006B, Frisancho-Kiss 2007, Fairweather 2012A). Elevated myocarditis in males is not due to increased viral replication in the heart, which is not significantly different between the sexes (Frisancho-Kiss 2006B, Frisancho-Kiss 2007). Male

BALB/c mice with CVB3 myocarditis develop a predominantly Th1-type adaptive immune response with more Th1-induced IgG2a anti-CVB3 antibodies compared to female mice, who develop a dominant Th2 response and more $Foxp3^+$ Treg cells (Huber 1994, Huber 1999, Frisancho-Kiss 2006A, Frisancho-Kiss 2007, Huber 2008, Frisancho-Kiss 2009). Huber et al have found that $V\gamma4^+$ γδT cells are increased in the heart of male mice drive a Th1-type immune response (Huber 1994, Huber 2005, Huber 2008). Using the hybrid CVB3 model, the dominant Th1-type immune response in male BALB/c mice was found not to be due to classical IL-12/ STAT4-induced interferon IFNγ production, but instead to be due to TLR4-induced IL-18- a cytokine that strongly induces IFNγ (Frisancho-Kiss 2006B). Patients with clinically suspected myocarditis or DCM were found to have higher levels of IFNγ and these patients were mainly men (Warraich 2011).

CD11b/CR3+ immune cells are the dominant infiltrating cells in the heart of male BALB/c mice with CVB3 myocarditis and in male myocarditis patients (Frisancho-Kiss 2007 Frisancho-Kiss 2009, Fairweather 2014). CD11b is expressed on macrophages, mast cells, neutrophils and some dendritic cell populations, for example. Male BALB/c mice also have more classically activated M1 macrophages in the heart during acute CVB3 myocarditis, while females have more inhibitory alternatively activated M2 macrophages (Fairweather 2009, Abston 2012A). In the hybrid CVB3 model TLR4 is expressed on macrophages that resemble a M2- or MDSC-phenotype more than a M1 phenotype (Frisancho-Kiss 2006A, Frisancho-Kiss 2007, Frisancho-Kiss 2009). Elevated CD11b and TLR4 expression on macrophages and mast cells from male BALB/c mice with CVB3 myocarditis are thought to play a key role in increasing IL-1β levels in the heart and driving cardiac remodeling and fibrosis in males (Fairweather 2003, Frisancho-Kiss 2007,

Fairweather 2008B, Levick 2011, Coronado 2012, Abston 2012A, Abston 2012B). CD11b and TLR4 mRNA expression has been found to be higher in male myocarditis and DCM patients (Satoh 2003, Satoh 2004, Fairweather 2014). During the innate immune response to heart-passaged CVB3 in the spleen, male BALB/c mice were found to upregulate genes associated with promoting CVD (Onyimba 2011). Compared to female BALB/c mice, males have increased TLR4 expression on macrophages and mast cells in the spleen at 12 hours post infection (pi) (Frisancho-Kiss 2006A). A recent study in mice showed that although sex chromosomes influence CVB3 myocarditis, sex hormones mediate the dominant sex effect (Robinson 2011). Limited studies in myocarditis patients exist and so most of our understanding on the role of sex hormones comes from studies using myocarditis models in mice.

Sex differences in cardiac remodeling, DCM and heart failure

Heart failure occurs with advancing age in both sexes (Regitz-Zagrosek 2011). Even so, men are at an increased risk for developing heart failure (Cleland 2003, Robinson 2011). Heart failure can be the consequence of atherosclerosis, myocarditis, and DCM, which are all male dominant CVDs (Sliwa 2000, Sliwa 2006, Fairweather 2012A). Sheppard et al found that men with myocarditis or acute DCM had higher cardiac expression of genes associated with apoptosis that correlated to increased risk of heart failure compared to women (Sheppard 2005). In contrast, heart failure with preserved ejection fraction is more common in women than men (Bursi 2006). However, age is an important factor in heart failure cases in women (Regitz-Zagrosek 2011). Women develop heart failure at an older age than men.

Regardless of age, women with heart failure have better heart function than men (Lenzen 2008, Barsheshet 2012).

Cardiac remodeling is a critical step in the progression from myocarditis to DCM and heart failure (Asakura 2009, Kania 2009). Cardiac remodeling involves the break down and synthesis of extracellular matrix component. This process is regulated by enzymes and cytokines released from immune cells, and mast cells and macrophages in particular (Fairweather 2008A, Fairweather 2008B, Levick 2011, Coronado 2012). Serpin A 3n, also known as α1-anti-chymotrypsin, is released from mast cells and has been found to be upregulated in 11 separate microarray studies of acute DCM patients (Asakura 2009). This gene was also one of the most highly expressed genes by microarray in male BALB/c mice with acute CVB3 myocarditis (Coronado 2012). Profibrotic cytokines that promote remodeling and fibrosis during myocarditis/ DCM include IL-4, IL-1β, IL-33, IL-17, and TGFβ_1 (Fairweather 2004A, Fairweather 2006, Baldeviano 2010, Coronado 2012). Only male mice that have higher numbers of mast cells and respond to CVB3 infection with a "mixed Th1/Th2"-type immune response go on to develop DCM and heart failure (Fairweather 2004A, Fairweather 2006, Coronado 2012, Abston 2012A, Abston 2012B, Abston 2013). This includes BALB/c and A/J mice. CVB3 myocarditis in male mice is far more severe if driven by the Th2 cytokine IL-33 compared to the Th2 cytokine IL-4 (Abston 2012A, Abston 2012B). Female BALB/c mice develop only very mild DCM after myocarditis (2/10 BALB/c females have mild DCM compared to 10/10 males that have severe DCM) (Coronado 2012). Similarly, men with myocarditis were 2x more likely to present with myocardial fibrosis by cardiac magnetic resonance imaging then women (Cocker 2009). Several studies report that men with myocarditis or DCM have a greater

induction of extracellular matrix proteins and/or fibrosis in the heart compared to women (Haddad 2008, Cocker 2009, Regitz-Zagrosek 2011). Studies in animal models suggest that testosterone is responsible for increasing cardiac remodeling in males (Cavasin 2006). Estrogen signaling via ERβ has been found to prevent cardiac fibrosis in females by blocking TGFβ1 and collagen synthesis (Hammes 2011). Overall, these studies suggest that elevated testosterone in men increases remodeling and fibrosis, leading to DCM and heart failure.

Vitamin D

Vitamin D (VitD) regulates serum calcium levels and bone homeostasis, but also functions as a sex steroid. VitD is obtained from cholesterol in the skin and exposure to ultraviolet B (UVB) from the sun or from dietary sources forms pre-vitamin D, which is hydroxylated in the liver by vitamin D 25-hydroxylase (Cyp2R1) and carried in the bloodstream by VitD binding protein (DBP) to the kidney where it is converted by 25-dihydroxyvitamin D3-1α-hydroxylase (Cyp27B1) to the active form of VitD (i.e., 1a,25-dihydroxyvitamin D3), which binds the VitD receptor (VDR) (Lavie 2011, Plum 2010, Zittermann 2010, Zhu 2013, Christakos 2016). VitD bound to the VDR forms a complex with the retinoid X receptor and this complex activates VitD response elements (VDREs) that influence a large number of genes at a time in a manner similar to estrogen and androgen response elements (Lisse 2011, Trochoutsou 2015). Additionally, many genes that regulate immune function have a VDRE in their promoter, like tumor necrosis factor for example (Bikle 2011). VitD is regulated in part by 1,25-dihydroxyvitamin D3 24-hydroxylase (Cyp24A1), which actively down-regulates VitD levels (Zittermann 2010). Many tissues are able to produce VitD and express the VDR including cardiomyocytes, fibroblasts, vascular

endothelial cells, and immune cells (Norman 2006, Mason 2013). Macrophages possess all of the components necessary to import/ synthesize cholesterol and convert it to active VitD including Cyp2R1 and Cyp27B1 (Bikle 2011, Onyimba 2011). Although VitD is mainly known for its role in promoting bone health it has been shown to influence innate immune signaling (Liu 2009, Lee 2011, Gambhir 2011, Verway 2013), suggesting that it could exert sex-specific effects on inflammation.

VitD/ VDR is known to mediate protection against infections where it is able to activate mast cells, macrophages, and dendritic cells (Bikle 2011). In response to infection, VitD upregulates TLR2, TLR4, CD14 (part of TLR4 signaling complex), the inflammasome, IL-1β, TNF and IFNγ, for example (Rook 1986, Wang 2004, Liu 2006, Baroni 2007, Liu 2007). In tissue culture studies and animal models, VitD has been shown to increase Th2 immune responses, IL-10, Treg, alternatively activate M2 macrophages, and TGFβ (Gregori 2001, Topiliski 2004, Daniel 2008, Bikle 2011, Litwack 2011). These results appear contradictory, but investigators in these studies did not describe the sex of animals or cells used in their experiments. Because VitD is a sex steroid, sex differences in its effect on immune cells are likely to exist. Although few studies examine the issue of sex differences in VitD, one study did report that VitD administration was only protective in female mice in an animal model of multiple sclerosis, an autoimmune disease that occurs more frequently in women than men (Spach 2005, Fairweather 2008A).

Vitamin D deficiency and autoimmune disease

Deficiency in the active form of VitD (i.e., 1α,25-dihydroxyvitamin D3) is highly prevalent worldwide with around 25% of the population in the US found to have inadequate

VitD levels and 8% at risk for deficiency (Plum 2010, Lavie 2011, Looker 2011, Litwack 2011, Hilger 2014, Welles 2014, Alkerwi 2015). VitD deficiency is defined by the Institute of Medicine as <10 ng/mL based on the role of VitD in bone health (Lavie 2011). Many published studies consider VitD levels <20 ng/mL to be deficient and levels <30 ng/mL as inadequate because they have found an association with disease with VitD levels <20 ng/mL (Lavie 2011, Looker 2011, Ross 2011, Welles 2014). A number of epidemiologic studies have found an association between low levels of VitD and all-cause mortality (Melamed 2008) and an increased risk of developing an autoimmune disease (Cutolo 2009, Ascherio 2010). However, whether low VitD levels cause disease or occur as a result of the disease process is not yet clear.

One autoimmune disease where low serum VitD levels have been associated with increased risk of disease is systemic lupus erythematosus (SLE) (Muller 1995, Cutolo 2011). One factor that could complicate interpreting this data is that SLE patients are recommended to avoid sunlight and use sunscreen to prevent rashes associated with the disease. Also, SLE occurs more frequently in dark-skinned women, and dark skin is known to lower circulating VitD levels (Clemens 1982, Cutolo 2011, Tang 2012). Also, kidney dysfunction is a primary component of SLE (Kronbichler 2013), and this is a key organ that metabolizes VitD to its active form which could also result in lower circulating VitD levels. Evidence that VitD may protect against SLE comes from animal studies where lupus-prone MRL/lpr mice given VitD had improved disease (Lemire 1992, Schoenfeld 2009).

VitD deficiency or insufficiency has also been found to occur more often in rheumatoid arthritis (RA) patients. In RA lower VitD levels have been found to correlate to higher disease activity (Oelzner 1998, Cutolo 2006). Additionally, RA patients treated with

VitD have improved disease (Andhelkovic 1999, Cutolo 2011, Zold 2011). Animal models of RA report that VitD administration reduces disease progression and severity (Cantoma 1998, Larsson 1998). VitD supplementation has been used to treat psoriatic arthritis (Huckins 1990, Gaal 2009, Cutolo 2011). Interestingly, a VDR polymorphism in psoriasis and psoriatic arthritis patients has been found to reduce success with VitD therapy (Datangac-Erden 2007). Overall, these data suggest that VitD reduces RA and psoriatic arthritis.

Scleroderma, also called systemic sclerosis, is a chronic autoimmune disease characterized by diffuse skin fibrosis and vasculopathy. Low circulating VitD levels are frequently observed in scleroderma patients. A number of studies report VitD insufficiency in 63-86% and deficiency in 35-95% of these patients (Calzolari 2009, Vacca 2009, Caramaschi 2010). Additionally, patients with VitD deficiency have worse disease than those that were VitD insufficient. Dermatologists use the topical form of VitD to treat scleroderma, demonstrating that VitD can decrease disease. TGFβ is believed to be responsible for increasing fibrosis in scleroderma patients. VitD administered to murine cells has been found to inhibit TGF production (Zold 2008, Zold 2011, Artaza 2009). Again, sex of the cells, animals or patients was not addressed in these studies.

Another autoimmune disease that has been linked to low VitD levels is Sjögren's syndrome, which is an inflammation of the salivary and lacrimal glands that results in dry mouth and eyes (Kronbichler 2013, Brandt 2015). Several studies have found that low VitD levels in Sjögren's syndrome patients correlate with increased disease severity (Muller 1990, Bang 1999). Additionally, low VitD levels correlated with higher levels of rheumatoid factor, which are antibodies made against antibody that often lead to IC formation. It is possible that

low VitD levels in Sjögren's syndrome patients may be due to kidney damage caused by the disease potentially affecting the body's ability to synthesize VitD (Kronbichler 2013).

Vitamin D deficiency and cardiovascular disease

Epidemiologic studies have found that VitD deficiency is associated with an increased risk of CVD (Agarwal 2011, Lavie 2011, Welles 2014). CVDs with this association include hypertension (Forman 2007, Forman 2008), myocardial infarction (Marniemi 2005, Giovannucci 2008, Brøndum-Jacobsen 2012), peripheral artery disease (Melamed 2008), stroke (Marniemi 2005, Poole 2006), congestive heart failure (Schleithoff 2006), heart failure and all-cause mortality (Dobnig 2008, Wang 2008, Ginde 2009, Kilkkinen 2009, Agarwal 2011, Tomson 2013, Belen 2015, Lutsey 2015). Two recent studies reported that women with low VitD levels were at an increased risk for myocardial infarction compared to men (Karakas 2013, Verdoia 2015). Whether the association of low VitD levels with increased risk of CVD is due to low exposure to sunlight or poor nutrition, for example, or directly related to the pathology of CVD remains unclear. There are no reports on the role of VitD on myocarditis or DCM.

Endocrine Disruptors

The term endocrine disruptor (ED) was first described in 1991 by a group of scientists who gathered in order to discuss what could be causing adverse effects in the offspring of wild animals. Then in 1995 the Environmental Protection Agency (EPA) defined endocrine disruptors as "exogenous agents that interfere with the synthesis, secretion, transport, binding, action, and elimination of natural hormones (like estrogen) in the body that are

responsible for the maintenance of homeostasis, reproduction, development, and/or behavior" (Kavlock 1997, Crisp 1998, Vandenberg 2009). There are a variety of agents that have been classified as EDs including metals, pesticides, estrogens from plants, fungi, and synthetically made chemicals like bisphenol A (BPA) (Ahmed 2000, Darbre 2006, Rahman Kabir 2015, Jochmanova 2015). Estrogen has been found to increase transport of TLRs from the endoplasmic reticulum and to increase T cell and B cell activation (Panchanathan 2008, Cunningham 2011, Panchanathan 2013, Kharrazian 2014), suggesting that EDs may also influence the immune system. Using a gene expression assay, bisphenol S (BPS), the replacement for BPA in many products, was found to effect 1,980 genes of which 900 were upregulated compared to BPA which only altered 48 genes (Fic 2015). Interestingly, the genes that were altered by BPA were mostly immunological in nature (Fic 2015).

Bisphenol A

BPA or 2,2-bis(4-hydroxyphenyl)propane (CAS No. 80-05-7) was first synthesized in 1891 by a Russian scientist Aleksander Dianin (Michalowicz 2014, Acconcia 2015). BPA is made by using a catalyst to condense acetone and phenol. Importantly it is moderately water soluble, which is considered to be a primary exposure route in the population (US EPA 2014). The half-life of BPA in humans is $t^{1/2}$ 5.3 hours and it is metabolized mainly by glucuronidation, but can also to be metabolized by sulfation (Corrales 2015). In 1930, BPA began to be used as a synthetic estrogen to increase estrogen levels in women that did not produce estrogen naturally to prevent miscarriages. After that it was found that BPA also has properties that allow its use in plastics, which began in the 1950s and continues to this day (Michalowicz 2014, Acconcia 2015). In 2004, 1 million tons of BPA was produced in the US

(US National Toxicology Program 2008). In 2009 it was estimated that 6 billion pounds of BPA was produced worldwide (Vandenberg 2009). BPA or its metabolites have been found in the urine of 90% of the US population and in the blood of 97% of pregnant mothers (Calafat 2005, Wolff 2007, Calafat 2008, Jenkins 2011, Bauer 2012, Koch 2012, Gerona 2016, Han 2016). Some studies have reported that higher levels of BPA (4.61 ng/mL) have been found in pregnant women from low income households (Gerona 2016). BPA can be transferred to the fetus through the placenta and the milk of the mother (Mead 2008, Balakrishnan 2010). The BPA level in babies is also a public health concern as babies have low expression of the enzymes needed for BPA metabolism.

BPA was initially used in plastics in order to allow plastic to be used multiple times (i.e., to allow recycling) (Mirmina 2014). BPA is used in a number of different types of plastic such as polycarbonate, polysulfone, and polyphthalate carbonate. Importantly from a toxicological perspective, BPA is released into solution from polycarbonate after heating of plastic (water) bottles or food containers and/or through direct UV exposure by sunlight (Rowell 2016). Plastic items labeled with recycling codes 3 and 7 contain BPA (Mirmina 2014). BPA is found in a number of widely used products including plastic water bottles, epoxy resins that line food and beverage cans, plastic food containers, receipts, dental amalgams, water pipes, paper products like photocopy paper, medical devices, cigarettes, toys, baby teethers, and residual industrial and waste found in the environment (Olea 1996, Snyder 2000, Matthews 2001, Howdeshell 2003, Hunt 2003, Jenkins 2011, Belcher 2012, Teeguarden 2013, Mirmina 2014, Oldring 2014, Berger 2015, Corrales 2015, Lorber 2015, Rahman Kabir 2015, Yama 2015, Gerona 2016, Hehn 2016, Liao 2016, Ndaw 2016). BPA and the BPA replacements BPS and bisphenol F (BPF) have been detected in 75% of foods

in the US such as fruits, dairy products, and "preserved" foods (Liao 2013). BPA and BPS were detected in 98% of thermal paper receipts tested in Brazil (Rocha 2015). BPA has also been detected in water, soil, air, landfills, sewage sludge, electronic waste, and wildlife (Corrales 2015, Rahman Kabir 2015).

There has been a lot of controversy about what is defined as a "low dose" of BPA. Toxicology studies define low doses as the dose that causes the lowest adverse effect level (LOAEL) (50,000 μg/kg/day) (US National Toxicology Program). A low dose exposure can be defined in two separate ways: 1) as any dose below the LOAEL (50,000 μg/kg/day) and 2) the "environmental relevant dose" which is the level of chemical that is present in the blood in a population (Vandenberg 2012, Acconcia 2015, Vandenberg 2014). BPA has been discovered to have a dose response curve that is an inverted "U", which indicates a non-monotonic dose response curve. This means that low doses of BPA are associated with increasing disease while higher doses protect against disease up to a certain very high dose (Nagel 1997, von Saal 2005, Vandenberg 2012, Acconcia 2015, Zoeller 2015). If BPA follows a non-monotonic dose response curve, then extrapolations from the high doses used in toxicity testing to very low doses experienced by humans would not be an accurate measure of exposure risk (Zoeller 2015).

BPA has been found to have estrogenic effects in multiple estrogen assays (Andersen 1999). BPA can induce effects by both genomic means (i.e., ERs binding to the ERE) and non-genomic means (e.i., signaling pathways like the MAP kinase pathway) (Goodson 2004, Marino 2012, Acconcia 2015). BPA influences ERs by binding to the receptor and causing it to be in its active formation even when estrogen is not present (Li 2015). Studies have shown that although BPA binds to ERα, that the molecular bonds (i.e., hydrogen bonds and van der

Waals forces) are much weaker than when estrogen binds ERβ (Li 2015). Although BPA binds to ERs with a lower affinity than estrogen, one way in which BPA could cause transcriptional changes through the ERs and activation of ERE is by inducing conformational changes of the ERs that lead to either agonist or antagonist functions or by altering its binding to other transcription factors or acting as a co-activator (Ascenzi 2006, Acconcia 2015). 17β-estradiol (E2) binds to both ERα and ERβ, but it has been found that it does not bind to ERRγ. No endogenous ligand is yet known for ERRγ, but BPA does appear to target the receptor. E2 is known to bind with a higher affinity to ERα than to ERβ (Kuiper 1997, Zaid 2015). BPA binds to both ERα and ERβ, but at a 10,000 times lower potency than E2. Additionally, BPA has been found to have a higher affinity for ERβ than ERα, the opposite of E2 (Kuiper 1997, Hiroi 1999, Molina-Molina 2013, Li 2015, Zaid 2015).

The first studies on the effect of BPA in animal models discovered that BPA significantly affected reproductive tissues (Cabaton 2011, Markey 2011, Vandenberg 2013, McGuinn 2015). Since then BPA has been associated in epidemiology and animal studies with increasing a number of diseases including cancers (Jenkins 2011 Costas 2015), diabetes in both prenatal and adult exposures (Melzer 2010, Bodin 2014, Aekplakorn 2015), obesity in both children and adults (Carwille 2011, Trasande 2012, Ranciere 2015, Yang 2016), and asthma/ wheezing (Nakajima 2012, Spanier 2012, Peters 2013). Epidemiology studies have also found associations between BPA and cardiovascular conditions including coronary artery disease, peripheral artery disease, high blood pressure, and hypertension (Melzer 2012, Ranciere 2015, Shankar 2012, Gao 2014, Han 2016). Rodent models have found that BPA has an effect on the heart including atherosclerosis, arrhythmias, blood pressure, vascular inflammation, ventricular contraction, and cardiac remodeling (Yan 2011, Patel 2013, Gao

2013, Gao 2014, Kim 2014, Belcher 2015). BPA was found to alter electrical signals in the heart in a rat model, and at very high doses could cause complete heart block (Posnack 2014). No studies have assessed the effect of BPA on myocarditis or DCM.

Epidemiology and animal models have also found an association between BPA and pyometra (infection of the uterus), asthma, and type II diabetes (Kendziorski 2012, O'Brien 2014). BPA was able to induce eosinophilia in the alveoli of the lung and increase IL-1β expression in the ovalbumin model of asthma in mice (He 2016). In another study BPA exposure led to increased reactive oxygen species and IL-1β in the lung (Yang 2015). BPA has been found to be associated with an increased prevalence of multiple sclerosis in humans (Orton 2004, Weinstock-Guttman 2003, Jochmanova 2015) and to increase disease in animal models (Brinkmeyer-Langford 2014, Jochmanova 2015). Prenatal exposure to BPA was found to alter cytokine levels in cord blood immune cells stimulated with a TLR agonist (Liao 2016), indicating that BPA can have an effect on the immune response. In an animal model of SLE, BPA exposure led to increased IgM and anti-DNA autoantibody release from B1 cells (Yurino 2004) as well as IgA and IgG2 release from B cells (Goto 2007, Rogers 2013). Adult exposure to BPA caused histamine release from bone marrow-derived mast cells (O'Brien 2014). BPA was also found to decrease Treg cells in mice exposed either prenatally or as adults (Yan 2008, Rogers 2013).

Bisphenol S

Concerns over BPA exposure in the public led to the replacement of BPA in baby bottles and many plastic water/ drink bottles with another component of plastic called BPS (Dieter 2012). Fewer than 100 papers have been reported in PubMed that mention BPS. The

first paper mentioning BPS was published in 2000 and titled, "Estrogenic activity of dental materials and bisphenol-A related chemicals in vitro" by Hashimoto and Nakamura in the journal *Dental Materials* (Hashimoto 2000). Of the 99 papers reported in PubMed, over 50% have been published in the last two years and only 15 examine *in vivo* or *in vitro* effects of BPS.

Several studies assessing the estrogenic effect of BPS found that BPS can bind to ERs, but its estrogenic potency depends on the location of the ER (Rochester 2015). When BPS binds to the nuclear ER it was less estrogenic than BPA, but if it bound to membrane ERs it was more estrogenic than BPA (Rochester 2015). When E2 is bound to ERα, a high concentration of BPA (10μM) can displace E2 from its binding site. In contrast, BPS only inhibited about 70% of the binding of E2 compared to BPA (Molina-Molina 2013). BPA can activate both ERα and ERβ, whereas BPS more potently activates ERβ compared to ERα (Molina-Molina 2013). BPA and BPS are also thought to bind to the AR receptor as an agonist but at only 20% of the binding of testosterone (Molina-Molina 2013). However, BPS, but not BPA, can also act as an ER antagonist (Molina-Molina 2013). One study assessed the effect of BPS on CVD and found that BPS increases heart rate, arrhythmias, and induced Ca2+ handling changes via ERβ in females, but not in males (Gao 2015) similar to what was seen with BPA. No studies have assessed the effect of BPS on myocarditis or DCM.

Hypothesis and Specific Aims

*Based on the literature we **hypothesize** that environmentally-derived sex steroids (i.e., VitD, EDs) will alter acute CVB3 myocarditis in a sex specific manner.*

Aim 1: Examine the effect of the VDR on CVB3 myocarditis in male and female mice

Deficiency in the active form of VitD is highly prevalent worldwide with around 25% of the population in the US found to have inadequate VitD levels and 8% at risk for deficiency. VitD is well known for its role in regulating serum calcium levels and bone homeostasis, but few realize that it also functions as a sex steroid. The VDR is expressed on cardiomyocytes, fibroblasts, vascular endothelial cells, and immune cells. Importantly, macrophages express the VDR and possess all of the components necessary to import/ synthesize cholesterol and convert it to active VitD including Cyp2R1 and Cyp27B1. The critical role that macrophages play in driving sex differences in CVB3 myocarditis in males, suggests that VitD via the VDR could influence myocardial inflammation in a sex specific manner. Recently low VitD levels have been associated with an increased risk of a number of CVDs, but no one has examined the role of VitD/VDR in myocarditis. This led us to examine the role of VDR signaling using male and female mice deficient in the VDR to examine its role in relation to sex differences in acute CVB3 myocarditis. Mice were fed a high calcium diet to prevent the development of rickets. We did not directly examine the role of VitD in these experiments.

Aim 2: Examine the effect of the endocrine disruptor BPA on CVB3 myocarditis in male and female mice

There are a number of agents that have been classified as EDs including metals, pesticides, estrogens from plants, fungi, and synthetically made chemicals like BPA. Myocarditis in patients and in animal models displays profound sex differences in the acute inflammation and the chronic stage that involves DCM. Testosterone has been shown to

increase CVB3 myocarditis in male BALB/c mice, but the role of estrogen is less well defined in this model. The first step in this thesis was to examine the role of estradiol on CVB3 myocarditis in BALB/c female mice. We show in this thesis that ovariectiomy of female mice significantly increases acute CVB3 myocarditis, while estradiol supplementation reverses this. These findings indicate that estrogen does indeed protect female mice from CVB3 myocarditis. This led us to *hypothesize* that endocrine disruptors that are known to alter ER function like BPA could alter acute myocarditis in a sex-specific manner.

In this aim we exposed male and female BALB/c (a mouse strain that develops acute and chronic myocarditis/ DCM) and C57BL/6 (a mouse strain that develops acute myocarditis but not DCM) mice to BPA in their drinking water for 2 weeks before infecting with CVB3. BPA exposure continued from day 0 until harvest at day 10 pi, and we used soy-free food and bedding in all of these experiments to prevent that estrogenic influence on the experiments. We began the experiments in the traditional plastic cages and water bottles that almost all investigators are using for their experiments. Then we repeated experiments using glass cages and water bottles to eliminate that route of additional exposure. We were surprised to find how significant the effect of the plastic cages was on our experiments and this led to many experiments that we had not originally anticipated. That meant that the chronic stage of the disease has not yet been examined and will need to be examined in the future.

Aim 3: Examine the effect of the endocrine disruptor BPS on CVB3 myocarditis in female mice

BPS is the primary ED that has replaced BPA in many plastic products like water bottles and baby bottles. For this reason it was thought when we started this project that BPS exposure may be a more environmentally relevant human exposure than BPA. The large published literature on BPA that has come out in the past 2 or so years, indicates that BPA is still found in many products that the population is exposed to (i.e., receipts, photocopy paper), and so BPA remains an important contaminant. Because of the critical role of sex hormones in driving myocarditis and DCM in humans and animal models, we *hypothesized* that BPS would alter CVB3 myocarditis in a sex specific manner. For these experiments, we exposed female BALB/c mice to BPS in their drinking water for 2 weeks before infecting mice with CVB3. BPS exposure continued from day 0 until harvest at day 10 pi, and we used soy-free food and bedding in all of these experiments to prevent that estrogenic influence on the experiments. In order to prevent ED exposure from the caging we used glass cages and water bottles for these experiments.

Chapter 2

Materials and Methods

Animal Care Ethics Statement

Mice were maintained and used in strict accordance with the recommendations in the Guide for the Care and Use the Laboratory Animals of the National Institutes of Health. Mice were maintained under pathogen-free conditions in the animal facility at the Johns Hopkins School of Medicine and at Mayo Clinic Florida, and approval obtained from the Animal Care and Use Committee of Johns Hopkins University and Mayo Clinic Florida for all procedures. Mice were sacrificed according to the Guide for the Care and Use the Laboratory Animals of the National Institutes of Health to minimize suffering.

CVB3-induced Myocarditis Model

Male and female BALB/c (stock #651) or C57BL/6 (stock #664) 6-8 week old adult mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were maintained under pathogen-free conditions in the animal facility at the Johns Hopkins School of Medicine or the Mayo Clinic Florida animal facility. Approval was obtained from the Animal Care and Use Committee for all experiments at both institutions. Generally, 10 mice per group/ sex were used for experiments. Mice were inoculated ip with sterile phosphate buffered saline (PBS) or 10^3 PFU of heart-passaged stock of CVB3 on day 0 and acute myocarditis examined at day 10 pi, as previously described (Fairweather 2007, Myers 2013). CVB3 (i.e., Nancy strain) was originally obtained from the American Type Culture Collection (ATCC; Manassas, VA) and grown in Vero cells (ATCC), as previously described (Myers 2013).

Gonadectomy and Hormone Replacement Studies

Sexually immature 4-6 week old WT BALB/c male or female mice (Jackson Laboratory, Bar Harbor, ME) received a bilateral [males- gonadectomy (Gdx), females- ovariectomy (Ovx)] or sham operation as previously described (Frisancho-Kiss 2009, Coronado 2012, Fairweather 2014). Mice were given 2 weeks to recover from surgery before being infected ip with CVB3, which is considered "day 0". Immediately after the operation a control (Con), testosterone (Te) or estradiol (E2) 90-day slow-release pellet was implanted subcutaneously (sc) in the midscapula region (Klein 2000, Siracusa 2008, Coronado 2012, Fairweather 2014). Circulating testosterone levels generated from the pellet are around 10-15 ng/mL, similar to physiologic levels of 10 ng/mL for normal male mice (Klein 2000, Coronado 2012). The estradiol pellet maintains physiological circulating estrogen concentrations of 100-200 pg/mL in female mice following gonadectomy (Siracusa 2008). Successful gonadectomy in males was confirmed by examining serum testosterone concentrations using an ELISA kit (Cayman Chemicals, Ann Arbor, MI), according to (Easterbrook 2007). Successful gonadectomy in females was confirmed immediately after harvest using uterine horn weights because epithelial cells of the uterine horn require estrogen for cell survival (O'Brien 2006). After a 2 week recovery period from surgery the mice were inoculated ip with 10^3 PFU of heart-passaged stock of CVB3 on day 0 and acute myocarditis examined at day 10 pi, as previously described (Coronado 2012, Myers 2013).

VDR-/- Studies

Male and female (6-8 week old) heterozygous VDR deficient (VDR+/-) (B6.129S4-*Vdr*^{tm1Mbd}/J, stock #6133) mice were obtained from the Jackson Laboratory (Bar Harbor,

ME). VDR+/- mice were mated to produce homozygous VDR deficient (VDR-/-) and wild type (WT) (VDR+/+) mice and genotyped according to the PCR protocol provided by the Jackson Laboratory. VDR +/+ and -/- mice were fed a Ca^{2+}-supplemented diet (TD.96348, 20% lactose, 2% calcium, 1.25% phosphorus; Harlan Laboratories, Madison, WI) to prevent the development of rickets (Li 1997). WT VDR+/+ littermates were used for comparison to VDR-/- mice. Mice were inoculated ip with 10^3 PFU of heart-passaged stock of CVB3 on day 0 and acute myocarditis examined at day 10 or day 35 pi, as previously described (Coronado 2012, Myers 2013).

Endocrine Disruptor Studies

6-8 week old WT male and female BALB/c (stock #651) or C57BL/6 (BL/6) (stock #664) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Plastic cages and water bottles (i.e., traditional housing) or glass cages and water bottles were used to house the mice as determined by the experiment. The bedding (Envigo-Tekland, 7990.BG) and food (Envigo-Tekland, 2020X) used in the endocrine disruptor studies were soy and phytoestrogen-free in order to prevent inclusion of other naturally occurring endocrine disruptors in the experiments and were purchased from Envigo (Minneapolis, MN). In separate experiments, MilliQ water containing varying doses of endocrine disruptors (BPA or BPS) or control MilliQ water were given to mice dissolved in drinking water for two weeks prior to inoculation ip with 10^3 PFU of heart-passaged stock of CVB3 on day 0 and acute myocarditis was examined at day 10 pi, as previously described (Coronado 2012, Myers 2013). BPA exposure was continued from day 0 until harvest at day 10 pi (**Figure 1**).

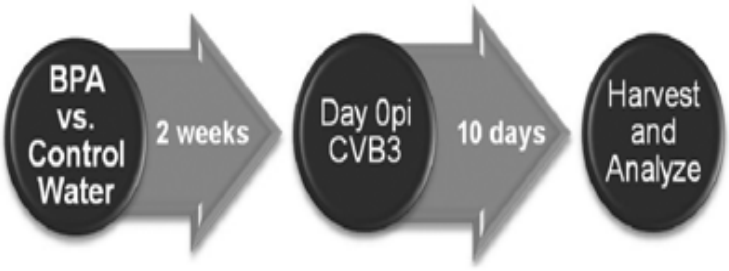


Figure 1. Timeline for BPA exposure experiments. BPA was dissolved in MilliQ water overnight at room temperature on a stir plate and serial dilutions were performed in order to obtain determined doses. BPA or control water was then placed in water bottles in mouse cages and mice were exposed for two weeks prior to infection with CVB3 at day 0 pi. BPA exposure in drinking water was continued until mice were harvested at day 10 pi for analysis. This same timeline was used for BPS experiments.

The BPA doses used in our studies, seen in **Table 1** below, were obtained from Jenkins et al and based on predicted daily exposure levels in the human population (Jenkins 2011). At the time of the development of this project Jenkins et al was the only study available that assessed the effect of BPA in a mouse model using oral exposure in drinking water. Estimated daily BPA intakes were determined previously by Jenkins et al (their unpublished data) by looking at how much water mice drink daily (Jenkins 2011). The EPA reference dose used in our experiments and reported by Jenkens et al was derived from EPA calculations. The EPA reference dose was calculated by using a safety factor of 1000x the LOAEL (lowest observable adverse effect level) (Vandenberg 2009). The EPA reference dose is defined as an estimate of the daily exposure to a susceptible individual without an appreciable risk of deleterious effects during a lifetime (EPA). The doses that were chosen are described in **Table 1** for BPA and **Table 2** for BPS, along with the estimated intake per mouse, and predicted human exposure level (Jenkins 2011). BPS dosing was determined by

analytical calculations using molecular weights of BPA and BPS to determine a BPA-equivalent dose for BPS.

Table 1. Bisphenol A (BPA) doses[a]

Treatment (μg BPA/L)	Estimated intake (μg BPA/kg BW)	Human exposure level
0	0	Control
2.5	0.5	Human relevant exposure
25	5	High human relevant exposure
250	50	EPA reference dose

[a] from Jenkins 2011

Table 2. Bisphenol S (BPS) doses[a,b]

Treatment (μg BPS/L)	Estimated intake (μg BPS/kg BW)	Human exposure level
0	0	Control
2.75	0.55	Human relevant exposure
27.5	5.5	High human relevant exposure
275	55	EPA reference dose

[a] Doses converted from BPA doses in Table 1 to their molar equivalent for BPS
[b] from Jenkins 2011

Myocarditis Patients

A diagnosis of myocarditis was confirmed by echocardiogram (Cooper 2009), and serum vitamin D3 levels were determined by liquid chromatography-mass spectrometry/ mass spectrometry (LC-MS/MS) at Mayo Clinic in Rochester, MN. Approval for use of the data was obtained from the Institutional Review Board (IRB) at both Mayo Clinic and Johns Hopkins University. Consent forms used under Mayo Clinic IRB approved protocols gave

permission for samples collected from patients at Mayo Clinic to be used for future research studies. Vitamin D levels were compared to patient ejection fraction (%EF) as a marker of cardiac function at baseline (time of patient diagnosis) and after treatment for disease (1 month, 2 months, 6 months, and 1 year). Vitamin D levels were defined by Institute of Medicine standards and common levels used in research studies (LaVie 2011, Ross 2011). The vitamin D status levels were defined as: deficient (<12 ng/mL), inadequate (Inadeq, 12-19 ng/mL), mildly deficient (Mild Def, 20-30 ng/mL), sufficient (30-50 ng/mL) or potentially harmful (50+ ng/mL). VitD levels were analyzed in the sera of lymphocytic myocarditis (LM) patients (*n*=16) and giant cell myocarditis (GCM) patients (*n*=28).

Histology

Mouse hearts were cut longitudinally and fixed in 10% phosphate-buffered formalin and embedded in paraffin for histological analysis. 5 μm sections were stained with hematoxylin and eosin (H&E) to detect inflammation, trichrome blue to detect collagen deposition (fibrosis), or toluidine blue to detect mast cell granules. Myocarditis was assessed as the percentage of the heart with inflammation compared to the overall size of the heart section using a microscope eyepiece grid, as previously (Fairweather 2003, Frisancho-Kiss 2009, Coronado 2012, Fairweather 2014). Sections were scored by at least two individuals blinded to the treatment group with 2 to 4 experiments conducted with 7-10 mice per group, in general.

Plaque Assay

The heart was homogenized at 10% weight/ volume in 2% minimal essential media and individual supernatants from hearts were used to preform plaque assays to determine the level of infectious virus, as previously described (Fairweather 2003, Frisancho-Kiss 2009, Coronado 2012, Fairweather 2014). Viral replication was expressed as the mean PFU/g of heart.

Quantitative Real-time PCR

RNA isolation of heart tissue

At harvest half of the heart was collected and stored at -80C for RNA isolation. Hearts were cut so that all chambers were present in both sections. Prior to RNA isolation frozen hearts were weighed. If individual hearts weighed more than 30 mg the heart was cut in half a second time prior to homogenization. Hearts were homogenized and lysed using Tissuelyser (Qiagen) with 7 mm stainless steel beads in RTL buffer with 0.5% DX buffer to reduce foam (Hilden, Germany). The homogenate was then placed in an automated RNA isolation and purification instrument, QIAcube, with reagents for RNase Easy Fibrous Mini Kit including a DNase and Proteinase K step (Qiagen). RNA was eluted into 30 μL. If the heart had been divided in the earlier step, the eluted RNA was pooled prior to being aliquoted. RNA quantification was determined in μg/μL using NanoDrop (Thermo Scientific, Waltham, MA).

qRT-PCR Method

Total RNA from mouse hearts was assessed by quantitative real time (qRT)-polymerase chain reaction (PCR) using Assay-on-Demand primers and probe sets and the ABI 7000 Taqman System from Applied Biosystems Biosystems (Foster City, CA) after RNA was converted to cDNA using High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems), as previously described (Onyimba 2011, Abston 2012A). Data are shown as relative gene expression (RGE) normalized to the housekeeping gene hypoxanthine phosphoribosyltransferase 1 (Hprt). Hprt was selected because GAPDH and actin, common housekeeping genes, were both found to be increased in expression during CVB3 myocarditis. Gene expression was analysed by assessing comparative quantification, which utilizes cycle threshold (Ct) for each primer to calculate the delta Ct (ΔCt), which is the threshold cycle comparison between the gene of interest and the housekeeping gene. This is then used to calculate the relative gene expression using the formula $RGE = 2^{-(\Delta Ct - \Delta Ct(max))}$.

Measurement of CVB3 genome levels by qRT-PCR

Probe sets to detect CVB3 were developed by Antoniak et al and obtained from Integrated DNA Technologies (Coralville, IA) (Antoniak 2013). Probe sets: CVB3 forward, 5′-CCCTGAATGCGGCTAATCC-3′; CVB3 reverse, 5′-ATTGTCACCATAAGCAGCCA-3′; CVB3 probe, 5′-FAM-TGCAGCGGAACCG-TAMRA-3′.

ELISA

The heart was homogenized at 10% weight/ volume in 2% minimal essential media and individual supernatants from hearts were used in enzyme linked immunosorbent assay (ELISA) to measure cytokine levels and in plaque assays to determine the level of infectious

virus, as previously described (Fairweather 2003, Frisancho-Kiss 2009, Coronado 2012, Fairweather 2014). Cytokines were determined using R&D Systems ELISA kits (Minneapolis, MN), according to the manufacturer's instructions and expressed as pg/g of heart. Hormone levels were determined using Cayman Chemical ELISA kits (Ann Arbor, MI, item #582701) and expressed as pg/mL of sera. Bisphenol A levels in the water were assessed using Abraxis-Ecologiena BPA ELISA kit (Warminster, PA) and expressed as μg/L water.

Western Blot

Heart tissues were dissected and snap frozen on dry ice and stored at -80 C until ready to be analyzed. Tissues were then lysed by homogenizing using a mechanical cell disperser with RIPA buffer (Abcam) and protease/phosphate inhibitor cocktail (BioRad) to obtain proteins from membrane, cytoplasm and nucleus. RIPA buffer contains 0.22% beta glycerophosphate, 10% tergitol-NP40, 0.18% sodium orthovanadate, 5% sodium deoxycholate, 0.38% EGTA, 1% SDS, 6.1% Tris, 0.29% EDTA, 8.8% sodium chloride, 1.12% sodium pyrophosphate decahydrate (Abcam). Extracted proteins were separated on a Criterion XT precast bis-tris 4-12% gel (BioRad) then transferred onto nitrocellulose membrane (BioRad). Samples were probed with primary antibodies specific for ERα (mouse IgG_1 monoclonal, H226, 1:200), phospho-ERα (rat polyclonal IgG, Serine 118, 1:200), ERβ (mouse monoclonal IgG_{2a}, 1531 1:200), phospho-ERβ (rat polyclonal IgG, Serine 87, 1:200), VDR (mouse poylclonal IgG_{2a}, D-6, 1:200) and normalized to Hprt (goat polyclonal IgG, N-15, 1:200). Species-specific secondary antibodies were conjugated to horseradish peroxidase (HRP) for densitometric analyses performed using Image J 1.50i.

BPA Analysis by Collaborators at University of Kentucky

In order to assess free BPA and BPA-glucuronide levels in the water and urine in our animal experiments we established a collaboration with Dr. Changcheng Zhou, Associate Professor in the Department of Pharmacology & Nutritional Sciences at the Saha Cardiovascular Research Center at the University of Kentucky. His laboratory found that BPA exposure to mice in a model of atherosclerosis increased disease and has developed a method to assess BPA levels in urine using modified chemical derivatization liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) instrumentation (Sui 2014). We sent Dr. Zhou urine samples collected from individual ear-tagged mice using the "free drop" method. Samples were collected on day 0, 3, 7, and 10 pi. After Dr. Zhou's laboratory performed the analysis, they sent us the free BPA and BPA-glucuronide levels expressed as ng/20μL.

Microarray Analysis

Processing and GeneChip analysis for microarray were performed on three heart samples for each treatment group (4 treatment groups/ timepoint: PBS inoculated uninfected male vs. female BALB/c mice and CVB3-myocarditis males vs. females at day 10 pi). Heart RNA (100 ng) was processed for hybridization to Affymetrix Mouse Gene ST 1.0 microarrays using the Affymetrix GeneChip Whole Transcript Sense Target Labeling Assay, according to manufacturer's protocol and a previously published method (Affymetrix, Santa Clara, CA) (Onyimba 2011, Coronado 2012). The Affymetrix Mouse GeneChip Gene 1.0 ST Array interrogates 28,853 well-annotated genes with 764,885 distinct probes. The expertise, facilities and instrumentation for Affymetrix GeneChip experimentation and analyses were

provided and supported by the Johns Hopkins Malaria Research Institute. Genes altered by CVB3 myocarditis and by sex were compared to known VDRE genes (Wang 2005). Candidate VDRE genes from the microarray analysis were verified by qRT-PCR. *Microarray data accession number.* The Affymetrix gene expression data were deposited to the GEO (www.ncbi.nlm.nih.gov/geo) with accession number GSE35182.

Echocardiography

In vivo cardiac function was performed by transthoracic echocardiogram using an Acuson Siemens Sequonia C256 and 13 megahertz (MHz) transducer on conscious mice. Nair was used to remove fur from mice prior to echocardiography in order to allow the transducer to have appropriate contact with the ultrasound gel on the mouse. The transducer was aligned in parasternal short axis view of the heart. A two-dimensional M-mode tracing was used to obtain left ventricular (LV) dimensions to determine fractional shortening (%FS) (measurement of the capacity of the LV) and relative wall thickness (RWT). A LV trace was preformed to assess chamber size (LVmass). These values were calculated by the Acuson Siemens Sequonia software automatically using formulas that can be found in Cihakova et al (Cihakova 2008).

Statistical Analysis

Normally distributed data comparing two groups were analysed using a 2-tailed, Student's *t* test. The Mann-Whitney rank sum test was used to evaluate nonparametric data. Data are expressed as mean ±SEM (standard error of the mean). A value of $p<0.05$ was considered significant.

When comparing more than 2 groups multiple comparison analysis was performed by ANOVA with each group compared to the corresponding control group. Data are expressed as mean ±SEM. A value of $p<0.05$ was considered significant.

Survival curves were analyzed using the Mantel-Cox test to determine if there was a significant difference in survival during chronic myocarditis. A value of $p<0.05$ was considered significant.

Correlation between x and y values was calculated by liner regression and shown as R squared (R^2) values. R is defined as the correlation coefficient (R^2 values closer to 1 indicate high correlation between values).

Analysis of microarray data was performed with Partek Genomics Suite (GS) Version 6.4 (Partek, MO, USA). Gene expression patterns for each gene were normalized to the median array intensity for all chips and data from infected animals were normalized to uninfected PBS controls (Onyimba 2011, Coronado 2012). Microarray data were analyzed with Partek GS software by 2-way ANOVA in order to look for significant differences between conditions (with sex and infection as factors) and then *P* values and fold changes were generated using Fisher's least significant difference (LSD) post hoc analysis for comparisons of diseased to undiseased or sex. False discovery rate (FDR) corrections for multiple comparisons (Benjamini-Hochberg) were applied to reduce the total number of false-positives. Genes were considered significant if they had a *P* value less than 0.05.

Chapter 3

Vitamin D Receptor Protects Females but Not Males from Myocarditis

Abstract

Studies estimate that 1 billion people worldwide have deficient or insufficient levels of VitD, while roughly 25% of individuals in the US have inadequate VitD levels. Epidemiology studies show that VitD deficiency is associated with an increased risk of cardiovascular disease, yet the mechanism remains unclear. Aside from its role in bone health, VitD functions as a sex steroid where it influences innate and adaptive immune cell function. In this study we examined the role of the VDR on viral-induced myocarditis using male and female knockout mice. Prior to infection the heart of female mice expressed significantly more VDR than males, and OVX with estradiol replacement revealed that elevated VDR in females was due at least in part to estrogen. In contrast, males expressed higher VDR levels during myocarditis, which was not due to testosterone. By microarray, 97 genes associated with the Vitamin D Response Element (VDRE) were upregulated in the heart of males during myocarditis compared to controls, while only 27 VDRE-related genes were upregulated in females, suggesting increased VDR signaling in males. VDR deficiency significantly increased myocarditis in female mice that typically have low cardiac inflammation. In contrast, VDR deficiency significantly reduced myocarditis in males. VDR deficient females developed a characteristically "male-type" immune response with increased TLR2, TLR4, caspase-1, IL-1β, IL-17, IFNγ, and classically activated M1 macrophage markers. These data suggest that women with low VitD levels may be at an increased risk for developing viral myocarditis similar to other autoimmune diseases, while activation of the VDR by viral infection may exacerbate disease in men by promoting innate inflammation. The data shown in this Chapter has been submitted for publication and that is why some Tables and Figures are given the description "Supplemental".

Introduction

Deficiency in the active form of VitD (i.e., 1,25-dihydroxyvitamin D3) is highly prevalent worldwide with around 25% of the population in the US found to have inadequate VitD levels and 8% at risk for deficiency (Plum 2010, Lavie 2011, Looker 2011, Litwack 2011, Hilger 2014, Welles 2014, Alkerwi 2015). VitD deficiency is most widely defined as less than 20 ng/mL, and inadequate VitD reported as less than 30 ng/mL (Lavie 2011, Looker 2011, Welles 2014). Epidemiologic studies report that VitD deficiency is associated with an increased risk of CVD (Agarwal 2011, Lavie 2011, Welles 2014), hypertension (Forman 2007, Forman 2008), myocardial infarction (Marniemi 2005, Giovannucci 2008, Brøndum-Jacobsen 2012), peripheral artery disease (Melamed 2008), stroke (Marniemi 2005, Poole 2006), congestive heart failure (Schleithoff 2006), and an increased risk of heart failure and all-cause mortality (Wang 2008, Dobnig 2008, Ginde 2009, Kilkkinen 2009, Agarwal 2011, Tomson 2013, Belen 2015, Lutsey 2015). Two recent studies reported that women with low VitD levels were at an increased risk for myocardial infarction compared to men (Karakas 2013, Verdoia 2015). Whether the association of low VitD levels with increased risk of CVD is due to low exposure to sunlight or poor nutrition, for example, or directly related to the pathology of CVD remains unclear. The role of VitD in myocarditis has not been previously investigated.

VitD obtained from exposure to the sun or from dietary sources is hydroxylated in the liver (and other organs) by 25-hydroxylase (Cyp2R1) and carried in the bloodstream by VitD binding protein (DBP) to the kidney where it is converted by 25-OH-D-1-hydroxylase (Cyp27B1) to the active form of VitD (i.e., 1,25-dihydroxyvitamin D3), which binds the VDR (Rowling 2007, Plum 2010, Zittermann 2010, Zhu 2013). VitD is regulated in part by

Cyp24A1, which actively down-regulates VitD levels (Zittermann 2010). VitD can also be produced locally in many different tissues and cell types including cardiomyocytes and vascular endothelial cells. Macrophages express VDRs and possess all of the components necessary to import/ synthesize cholesterol and convert it to active VitD including Cyp2R1 and 27B1 (Bikle 2011). VitD bound to the VDR forms a complex with the retinoid X receptor and this complex activates the VDRE, which influences a large number of genes at a time in a manner similar to estrogen and androgen response elements (Lisse 2011, Trochoutsou 2015). Although VitD is known for its role in promoting bone health, it is also a sex steroid that has been shown to influence innate immune signaling (Liu 2009, Lee 2011, Gambhir 2011, Verway 2013) suggesting that it could exert sex-specific effects on inflammation.

Myocarditis is an inflammation of the myocardium that can cause sudden death due to heart failure and may be responsible for up to half of all DCM cases in the US (Sagar 2012, Cooper 2014). DCM is a leading cause of chronic heart failure leading to the need for transplantation. Common viruses like influenza and CVB3 cause myocarditis in people and animal models (Cooper 2009, Fairweather 2012A, Fairweather 2012B, Myers 2013). Myocardial inflammation is far more severe in male BALB/c mice following CVB3 infection compared to females; and similar to patients, male mice progress from myocarditis to DCM and chronic heart failure while females recover (Huber 2005, Frisancho 2007, Cocker 2009, McNamara 2011, Coronado 2012, Fairweather 2013, Fairweather 2014). Previously we and others showed that genes related to the inflammasome including TLR4, caspase-1, IL-1β, IL-18 and IFNγ are elevated in the heart of male mice with CVB3 myocarditis leading to a dominant adaptive Th1-type immune response (Huber 1994, Fairweather 2003, Frisancho-

Kiss 2007, Coronado 2012, Roberts 2013). Gdx of male mice significantly reduces cardiac inflammation and converts males to a "female-type" immune response with increased Th2 cytokines (i.e., IL-4), Treg, inhibitory cytokines (i.e., IL-10), and more alternatively activated anti-inflammatory M2 macrophages (Frisancho-Kiss 2007, Frisancho-Kiss 2009, Li 2009). In this study we show that estrogen increases VDR expression in the heart of females, which reduces CVB3-induced myocarditis in females. In contrast, VDR activation in males after infection increases cardiac inflammation. These data suggest that women with VitD deficiency could be at an increased risk of developing viral myocarditis, while activation of the VDR by virus may exacerbate disease in men by promoting cardiodamaging innate immune pathways.

Results

Females express more VDR before infection, males during myocarditis

VDRs are expressed on a wide variety of cells including immune cells, vascular endothelial cells, cardiomyocytes and cardiac fibroblasts (Chen 2008, Plum 2010, Lavie 2011). Additionally, Cyp27B1 and Cyp24A1 have been reported to be expressed in the heart of rodents (Chen 2008). To determine whether components needed for VitD metabolism were present in the heart during murine CVB3 myocarditis, we compared mRNA levels of VDR, DBP, Cyp2R1, Cyp27B1 and Cyp24A1 in the heart of PBS-treated control male and female BALB/c mice vs. males and females with CVB3 myocarditis by qRT-PCR at day 10 pi (**Fig 1, Table 1**). We found that Cyp2R1 was significantly increased during acute myocarditis in both female (p=0.002) and male (p=0.002) mice compared to controls, but was significantly higher in males compared to females with myocarditis (p=0.03, **Table 1**). Cyp27B1, which is needed to convert VitD to its active form, did not increase during myocarditis, but was expressed at 2-fold higher levels in the heart than the housekeeping gene Hprt in both sexes (**Table 1**). Prior to infection, hearts from female mice had significantly higher expression of the VDR (p=0.008, **Fig 1A**) and DBP (p=0.04) compared to uninfected males (**Table 1**). In contrast, males with CVB3 myocarditis had increased expression of VDR (p=0.006, **Fig 1D**) and DBP (p=0.04, **Table 1**) compared to control males. VDR expression was not significantly different in females vs. males with CVB3 myocarditis (**Fig 1B**) or in females with myocarditis compared to control females (**Fig 1C**). However, the enzyme that breaks down VitD, Cyp24A1, was significantly higher in females vs. males with myocarditis (p=0.03, **Table 1**). Thus, the components needed to process VitD are present in the murine heart during myocarditis and differ between males and females.

Table 1. Expression of VDR-related genes in the heart of WT and mice with CVB3 myocarditis at day 10 pi

	PBS Female vs. CVB3 Female*			PBS Male vs. CVB3 Male		
	PBS	*CVB3*	*P value*	*PBS*	*CVB3*	*P value*
VDR	3.60±0.32	3.82±0.19	0.57	1.82±0.42	3.66±0.14	**0.006**
DBP	6.82±1.05	3.82±0.19	**0.04**	2.61±0.82	6.46±1.37	**0.04**
Cyp2R1	1.36±0.04	2.07±0.14	**0.002**	1.36±0.10	2.58±0.24	**0.002**
Cyp27B1	1.58±0.18	1.59±0.06	0.93	1.91±0.08	1.89±0.21	0.92
Cyp24A1	8.74±2.11	7.58±1.05	0.38	5.89±1.14	4.56±0.54	0.33
	PBS Female vs. Male			**CVB3 Myocarditis Female vs. Male**		
	Female	*Male*	*P value*	*Female*	*Male*	*P value*
VDR	3.60±0.32	1.82±0.42	**0.008**	3.82±0.19	3.66±0.14	0.54
DBP	6.82±1.05	2.61±0.82	**0.01**	3.82±0.19	6.46±1.37	0.07
Cyp2R1	1.36±0.04	1.36±0.10	0.96	2.07±0.14	2.58±0.24	**0.03**
Cyp27B1	1.58±0.18	1.91±0.08	0.13	1.59±0.06	1.89±0.21	0.22
Cyp24A1	8.74±2.11	5.89±1.14	0.26	7.58±1.05	4.56±0.54	**0.03**

* Data show relative gene expression normalized to the housekeeping gene hypoxanthine phosphoribosyltransferase 1 (Hprt) using qRT-PCR. Data show the mean +/-SEM of relative gene expression (RGE) compared to Hprt by qRT-PCR using a 2-tailed Mann-Whitney rank sum test, n=10/ group. Bold font indicates $p<0.05$.

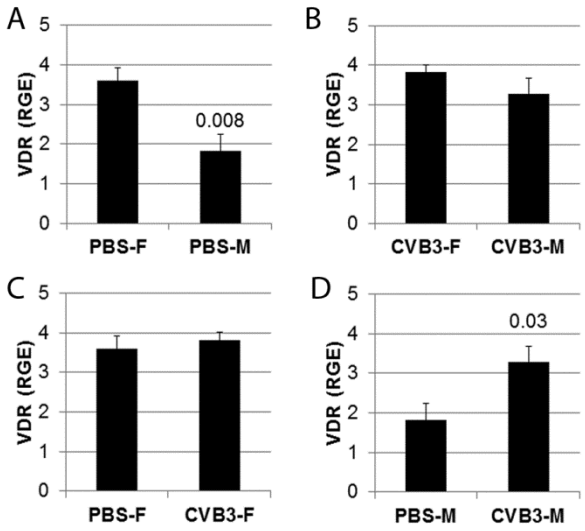


Figure 1. Greater VDR expression in female hearts prior to infection and male hearts during CVB3 myocarditis. Female (F) and male (M) BALB/c mice were injected with PBS or CVB3 ip on day 0 and the whole heart analyzed for VDR mRNA expression on day 10 pi, during acute myocarditis. Sex differences were examined in males and females **A)** without myocarditis, **B)** with CVB3-induced myocarditis, **C)** in females before and after disease, and **D)** in males before and after disease. Data show the mean +/-SEM of relative gene expression (RGE) compared to Hprt by qRT-PCR using a 2-tailed Mann-Whitney rank sum test, *n*=10/ group.

VDRE genes upregulated during CVB3 myocarditis

To gain a better understanding of genes related to VDR signaling that may be involved in CVB3 myocarditis, we conducted microarray analysis of undiseased (PBS-treated) male vs. female BALB/c mice compared to males and females with myocarditis. We picked genes to analyze based on published reports of genes found to be downstream of the VDRE (Wang 2005, Bosse 2009). Genes that are regulated by the VDRE that were increased in males with CVB3 myocarditis compared to control males include IFN-induced genes (*Ifit1-3*, 6.2-fold increase, *p*=0.0002), caspase-1 (2.2-fold increase, *p*=0.006), TGFβ2 (2.1-

fold increase, p=0.03), Cxcl11 (an M1 marker, 1.7-fold increase, p=0.04), and IL-1R antagonist (1.5-fold increase, p=0.02), for example (**Supplemental Table 1** and **Supplemental Fig 1**). The full list of up- and down-regulated VDRE genes in diseased and undiseased male and female mice are found in **Supplemental Table 1-4**. We found that 97 genes associated with the VDRE were significantly upregulated in the heart of male mice with myocarditis compared to control males (**Supplemental Table 1**), while only 27 VDRE genes were upregulated in females compared to undiseased females (**Supplemental Table 2**), suggesting that VDR signaling is greater in males with CVB3 myocarditis than females (**Fig 1D**).

Estrogen increases VDR expression in the heart of females

We showed previously that testosterone is responsible for increasing acute CVB3 myocarditis in male BALB/c mice (Frisancho-Kiss 2009, Coronado 2012). In this study we wanted to determine the role of estrogen (17β-estradiol) and testosterone on VDR expression in whole hearts during CVB3 myocarditis by qRT-PCR. Female and male sexually immature 4-6 week old WT BALB/c mice received a Sham operation or bilateral ovariectomy (Ovx) for females or gonadectomy (Gdx) for males and mice were allowed to recover from the surgery for 2 weeks before infection with CVB3 ip on day 0. At the time of surgery mice received either an empty control pellet (Con) or a slow-release estradiol (E2) or testosterone (Te) pellet subcutaneously (sc). The release of hormones has been found to last for at least 90 days (Klein 2000).

We found that female mice with an ovariectomy develop significantly increased CVB3 myocarditis as represented by the percent inflammation (p=0.001) (**Fig 2**), with a

severity similar to WT males (i.e., 30-40% inflammation) (Frisancho-Kiss 2007, Coronado 2012). Male mice with a gonadectomy have significantly reduced inflammation (p=0.0003) (**Fig 2**), similar to the severity of WT females (i.e., 10-15% inflammation) (Frisancho-Kiss 2007, Frisancho-Kiss 2009). Estradiol treatment of ovariectomized females (Ovx+E2) significantly decreased myocardial inflammation (p=0.02) and increased VDR expression in whole hearts (p=0.005) compared to females with an operation but no hormone supplementation (Ovx+Con) (**Fig 2A**). Success of the operation and estrogen replacement was verified by a significant increase in wet uterine horn weight (p=$4x10^{-7}$) (**Fig 2A**). In contrast, testosterone treatment of gonadectomized males (Gdx+Te) did not significantly alter VDR expression in the whole heart (p=0.18) in spite of its ability to increase myocarditis (p=0.04) and circulating testosterone levels (p=$3x10^{-5}$) compared to males with an operation but no hormone treatment (Gdx+Con) (**Fig 2B**). Thus, estrogen (i.e., estradiol) increases VDR expression in the heart of females with CVB3 myocarditis, but testosterone is not responsible for increasing VDR expression in males when examining expression levels in the whole heart. However, we showed previously that testosterone replacement only increased the CD11b-associated gene TSPO if immune cells were isolated from whole hearts and then gene expression examined (Fairweather 2014). In future experiments we will need to evaluate the effect of testosterone on VDR expression levels from isolated cardiac immune cells during myocarditis. It is possible that estrogen is increasing VDR expression primarily on cardiac resident cells rather than immune cells in females with myocarditis.

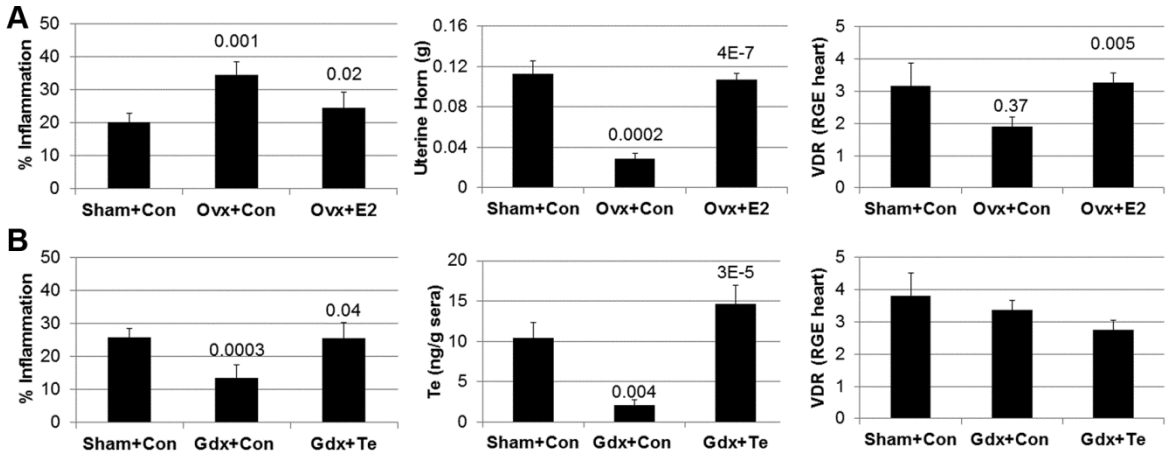


Figure 2. Estrogen increases cardiac VDR expression and decreases CVB3 myocarditis. A) Female and **B)** male sexually immature 4-6 week old WT BALB/c mice received a Sham operation or bilateral ovariectomy (Ovx) for females or gonadectomy (Gdx) for males and mice recovered from surgery for 2 weeks before infection with CVB3 ip on day 0. During surgery mice received either slow-release control pellets (Con), estradiol (E2) or testosterone (Te) sc. At day 10 pi, myocarditis (*left*), uterine horn weight or circulating Te levels (*center*), and cardiac VDR expression was assessed (*right*). Relative gene expression (RGE) of VDR by was compared to Hprt in hearts by qRT-PCR as Data show the mean +/-SEM using a 2-tailed Mann-Whitney Rank Sum test, *n*=7-10/group.

VDR reduces CVB3 myocarditis in females, but increases disease in males

Two recent studies found that low VitD levels increased the risk for myocardial infarction in women, but not in men (Karakas 2013, Verdoia 2015) suggesting a sex difference in the effect of VitD on CVD. However, no studies have been conducted examining the effect of VitD deficiency in human or mouse models of myocarditis. To assess the effect of VDR deficiency (-/-) on murine CVB3 myocarditis, VDR+/+ and VDR-/- littermates were provided a calcium supplemented diet from birth to prevent the development of cardiac dysfunction associated with rickets that would occur in knockout mice (Li 1997,

Chen 2011). Calcium supplementation continued throughout the experiment. 8-10 week old mice received CVB3 ip on day 0 and myocarditis was assessed histologically at day 10 pi, during acute myocarditis, as depicted in representative photos in **Figure 3A**. We found that VDR-/- females had significantly increased (p=2x10^{-8}) while VDR-/- males had significantly reduced (p=0.03) myocarditis at day 10 pi compared to WT VDR+/+ mice (**Fig 3B**). These results demonstrate that VDR signaling decreases myocarditis in female mice but increases inflammation in males.

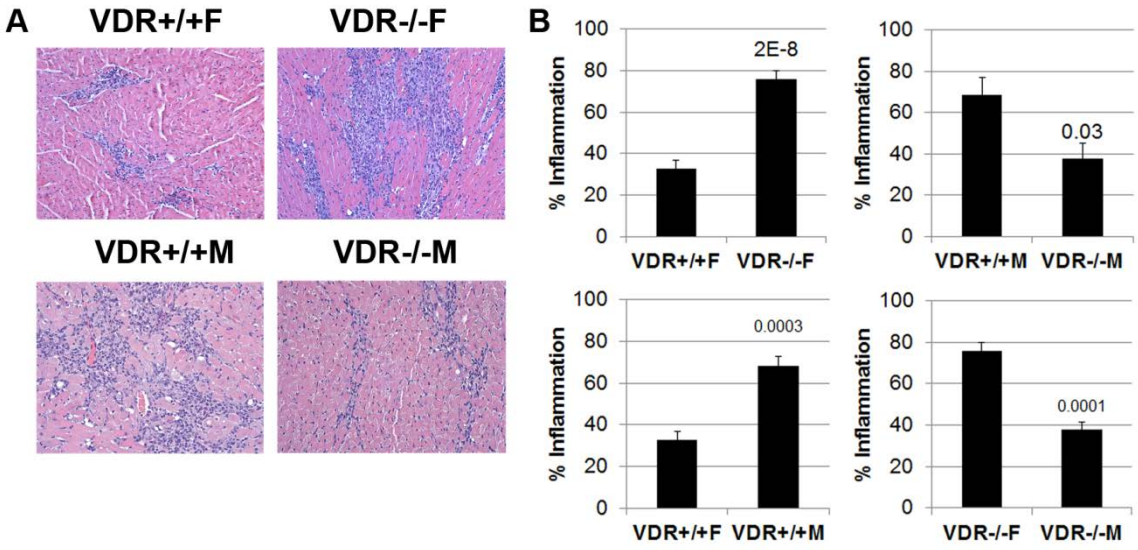


Figure 3. VDR deficiency increases myocarditis in females but decreases disease in males. Female (F) and male (M) WT+/+ and VDR-/- mice supplemented with calcium feed to prevent rickets from birth and injected with CVB3 ip on day 0 and myocarditis assessed at day 10 pi. **A)** Representative H&E photos of myocarditis, magnification x100. **B)** Average inflammation (i.e., myocarditis) assessed histologically showing the mean +/-SEM using a 2-tailed Mann-Whitney rank sum test, n=10-35/ group.

VDR deficiency does not alter viral replication in the heart

Because VitD/ VDR signaling is known to promote antimicrobial innate immune responses (Wang 2004, Wang 2010, Bikle 2011, Hewison 2011, Verway 2013, Tulk 2015) and to decrease certain viral infections like hepatitis C virus and HIV (Yano 2007, Campbell 2011), we examined whether VDR deficiency altered CVB3 replication in the heart during acute myocarditis. We found that VDR deficiency had no significant effect on viral replication in the heart during CVB3 myocarditis at day 10 pi (females: WT $2.5x10^{-4}\pm1.4x10^{-4}$, VDR-/- $2.7x10^{-3}\pm1.5x10^{-3}$ PFU/g heart, p=0.13; males: WT $7.3x10^{-2}$, VDR-/- $4.9x10^{-2}$ PFU/g heart, p=0.64; n=7-12/group). Thus, increased myocarditis in VDR-/- females was not due to increased viral replication.

VDR deficiency increases T cells in females

The VDR is known to be expressed on T and B cells, macrophages, mast cells, natural killer (NK) cells and Treg (Fairweather 2015, Toniato 2015). During CVB3 myocarditis male BALB/c mice have significantly higher cardiac inflammation consisting of CD11b+ immune cells including neutrophils, macrophages, and mast cells and mount a dominant Th1-type immune response, whereas females have higher B cells, T cells, Treg and mount a Th2-type immune response (Huber 2005, Frisancho-Kiss 2007, Frisancho-Kiss 2009, Roberts 2013, Fairweather 2014). In this study, the expression of markers for immune cells from whole hearts were assessed in WT+/+ and VDR-/- mice by qRT-PCR during acute myocarditis. VDR-/- females had significantly increased CD45 (p=0.02), CD3 (p=0.04), CD4 (p=0.04), and CD8 (p=0.04) compared to control females (**Fig 4A**), suggesting increased levels of T cells. VDR-/- males had increased Foxp3 (Treg) (p=0.04) and decreased cKit (market for mast cells and/or stem cells) (p=0.04) compared to control males (**Fig 4B**). We

also tested CD11b, CD14, GR1 and F4/80 expression levels and found no significant differences between VDR-/- and WT mice for either sex (data not shown). Both the increase in CD45 and T cell markers in VDR-/- females and the increase in the Treg marker FoxP3 and decrease in mast cell marker cKit in males are consistent with the histology findings (**Fig 3**).

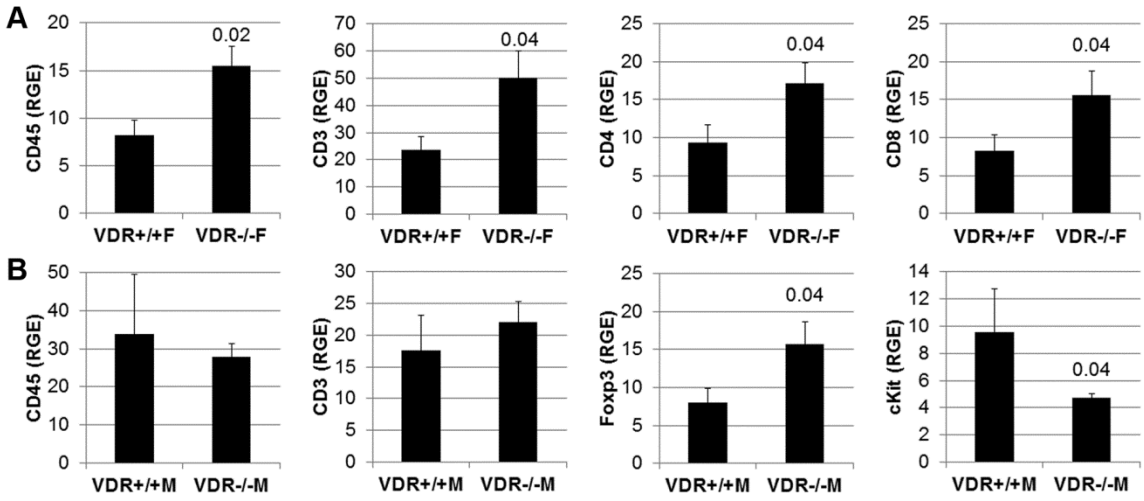


Figure 4. T cell markers increase in VDR deficient female hearts during myocarditis, Treg markers increase in males. A) Female (F) and **B)** male (M) WT (+/+) or VDR-/- mice were infected ip with CVB3 on day 0 and mRNA expression of immune cells examined in whole hearts at day 10 pi by qRT-PCR. Data show the mean +/-SEM relative gene expression (RGE) compared to Hprt by a 2-tailed Mann-Whitney rank sum test, *n*=7-10/group.

VDR deficiency increases IFNγ and IL-17 in females and IL-4 in males

VitD/ VDR signaling has been shown to increase Th2-type immune responses and Treg while decreasing Th1 and Th17 responses (Adorini 2008, Bruce 2010, Bikle 2011, Hewison 2011, Toniato 2015, Fairweather 2015). For this reason we examined IL-4, IFNγ, and IL-17 (IL-17A) levels in the heart of VDR +/+ and -/- mice with CVB3 myocarditis by

ELISA as an indicator of Th responses. We found that VDR-/- females had significantly increased IFN-γ (p=0.005) and IL-17 (p= 0.03) compared to control females (**Fig 5A**). In males, VDR-/- significantly decreased IFNγ (p=0.03) but increased IL-4 and IL-17 (p=0.04 and p=0.0002, respectively) compared to control males (**Fig 5B**). These results are consistent with the reported effects of VitD/ VDR for females but not for males because VitD is assumed to be protective for both women and men. These data demonstrate that sex differences exist in the effect of VDR on the immune response during viral myocarditis.

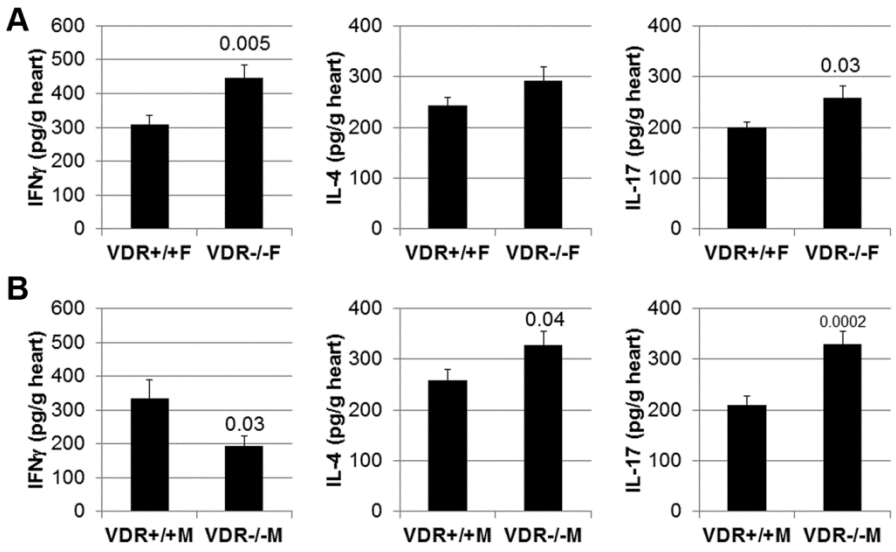


Figure 5. VDR deficiency increases cardiac IFNγ and IL-17 in female, but IL-4 and IL-17 in male hearts during CVB3 myocarditis. A) Female (F) and **B)** male (M) WT (+/+) or VDR-/- mice were infected ip with CVB3 at day 0 and cytokine levels representative of Th1 (IFNγ), Th2 (IL-4) and Th17 (IL-17) immune responses assessed in homogenized heart supernatants by ELISA at day 10 pi. Data show the mean +/-SEM pg/g of heart using a 2-tailed Mann-Whitney rank sum test, n=22-45/group.

VDR deficiency increases M1 macrophages in females and M2 in males

Classically activated M1 macrophages are driven by IFNs/Th1 responses and are increased in males during CVB3 myocarditis, while alternatively activated M2 macrophages are anti-inflammatory and are elevated in females with myocarditis (Li 2009, Fairweather 2009, Abston 2012A, Abston 2013). Markers of M2 macrophages include arginase-1 and Ym1 (eosinophilic protein from chitinase family) and M1 markers include the IFN-associated chemokines Cxcl9 and Cxcl10 (Fairweather 2009). Here we found that VDR-/- females had higher levels of M1 macrophage markers Cxcl9 (p=0.04) and Cxcl10 (p=0.04) compared to WT females (Fig 6A), whereas VDR-/- males had higher anti-inflammatory M2 macrophage markers arginase-1 (p=0.03) and Ym1 (p=0.03) compared to WT males (**Fig 6B**). Thus, VDR signaling increases M2 macrophages in females and M1 macrophages in males, which is consistent with the sex differences we observed in the shift of Th1/Th2-type cytokines mediated by VDR during myocarditis (**Fig 5**).

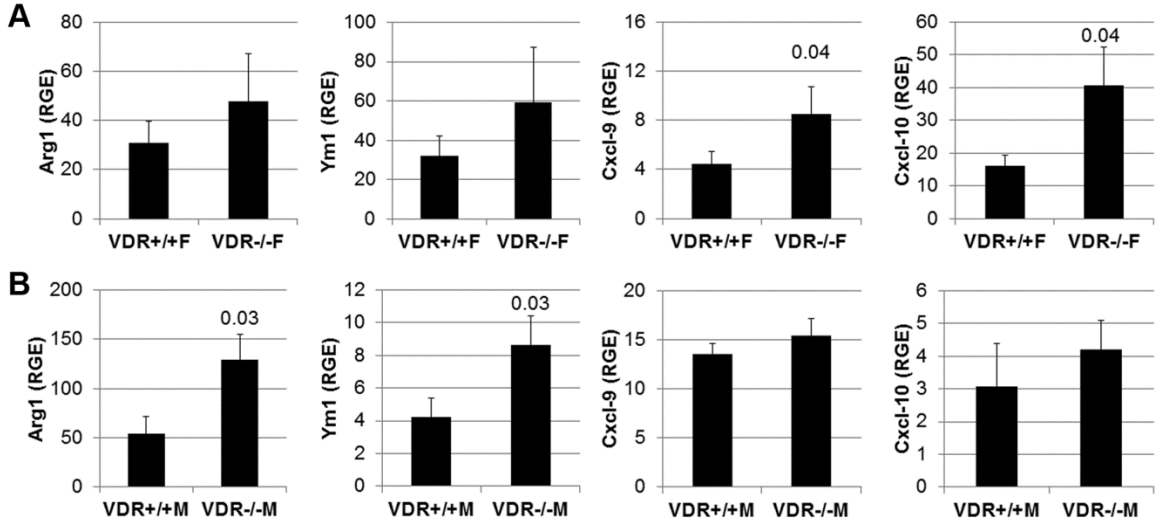


Figure 6. VDR deficiency increases cardiac M1 macrophages in females and alternatively activated M2 macrophages in males during myocarditis. A) Female (F) and **B)** male (M) WT (+/+) or VDR-/- mice were infected with CVB3 on day 0 and markers of M1 (Cxcl9, Cxcl10) vs. M2 (Arg1, Ym1) macrophages examined in whole hearts at day 10 pi by qRT-PCR. Data show the mean

+/-SEM of relative gene expression (RGE) compared to HPRT using a 2-tailed Mann-Whitney rank sum test, *n*=6-10/ group.

VDR deficiency activates TLR4/IL1R/capase-1/IL-1β pathway in females

We showed previously that components of IL-1R signaling including TLR4, caspase-1 and the inflammasome (i.e., TLR4, IL-1R, caspase-1, IL-1β and IL-18) are upregulated by testosterone during CVB3 myocarditis in male mice leading to more severe inflammation and DCM in males (Huber 2005, Frisancho-Kiss 2007, Frisancho-Kiss 2009, Coronado 2012, Roberts 2013, Fairweather 2015). In this study we found that VDR-/- females had significantly increased expression of TLR2 (p=0.02), TLR4 (p=0.03), caspase-1 (p=0.03) and IL-1R2 (p=0.03) by qRT-PCR, and increased active IL-1β (p=0.02) by ELISA compared to WT females (**Fig 7A**). In contrast, VDR-/- males had significantly decreased levels of IL-1β (p=0.02) compared to WT males (**Fig 7B**). These data indicate that VDR deficiency is driving females to a more "male-like" innate inflammatory profile during myocarditis. The decrease in IL-1β in males suggests that VDR increases this proinflammatory and profibrotic cytokine in males. Although we did not observe a change in TLR4 or caspase-1 in VDR-/- males compared to WT males, these components are required to produce active IL-1β and so must have been decreased earlier in the disease process. In future studies, earlier timepoints will be examined to confirm this conclusion.

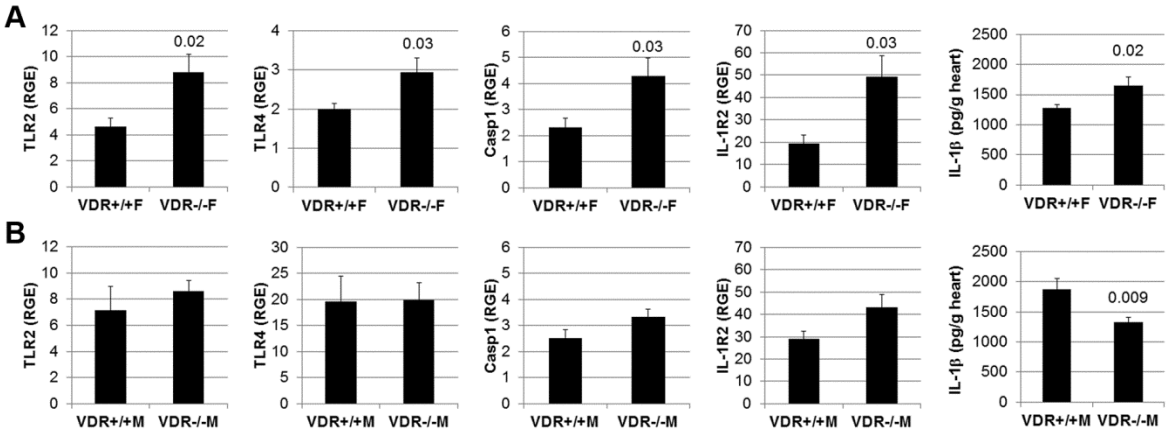


Figure 7. VDR deficiency increases cardiac TLR2/TLR4/IL-1R2/caspase/IL-1β pathway expression in females. A) Female (F) and **B)** male (M) WT (+/+) or VDR-/- mice were infected ip with CVB3 on day 0 and TLR2, TLR4, caspase-1 (casp1), IL-1R2 assessed by qRT-PCR and IL-1β by ELISA at day 10 pi. Data show the mean +/-SEM of relative gene expression (RGE) compared to Hprt for qRT-PCR (*n*=7-10/ group) or pg/g for ELISA using a 2-tailed Mann-Whitney rank sum test (*n*=22-45/ group).

VDR deficiency leads to hypertrophy and cardiac dysfunction during chronic myocarditis in males

VDR deficiency significantly decreased myocarditis in male mice (**Fig 3**), suggesting that VitD/ VDR increases myocarditis in males. In order to determine whether VDR altered the chronic phase of CVB3 myocarditis and DCM, we infected WT, VDR+/-, and VDR-/- male mice with CVB3 at day 0 and assessed myocarditis and DCM at day 35 pi. We were surprised to find that all of the VDR-/- mice died by day 32 pi, prior to assessment of heart function by echocardiography (**Fig 8**). We were not expecting this outcome because VDR-/- male mice had less acute CVB3 myocarditis, otherwise we would have performed

echocardiography at an earlier timepoint on these mice, as it is a non-invasive procedure. This will be done in future experiments.

In contrast, only one WT (VDR+/+) male mouse died during this time course (**Fig 8**). The surviving mice (VDR+/+ and VDR+/-) were then assessed at day 35 pi, during chronic myocarditis, by echocardiography to determine cardiac function (**Fig 9A**). VDR+/- mice had significantly increased hypertrophy as determined by increased left ventricular mass and relative wall thickness (RWT) (**Fig 9B&D**). Cardiac function was determined by the %FS and we found that the VDR+/- males had significantly lower %FS and decreased cardiac function (**Fig 9C**). It is possible that the deaths in the VDR-/- group were due to DCM and/or heart failure, but that will need to be assessed in future experiments. If VDR-/- mice developed DCM, the result would likely be due to the shift in VDR-/- male mice to a Th2/M2-type immune response which the Fairweather lab has shown leads to DCM and heart failure following myocarditis (Abston 2012A, Abston 2012B, Abston 2013).

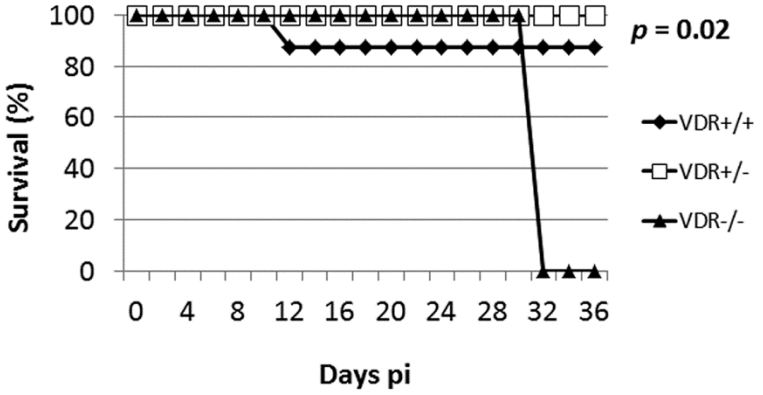


Figure 8. VDR deficiency leads to death in males during chronic myocarditis. Male WT (VDR+/+), heterozygote (VDR+/-) or VDR-/- mice were infected ip with CVB3 on day 0 and survival was tracked until day 35 pi when echocardiography was performed on surviving mice. Survival comparing the three groups was found to be significant using the Mantel-Cox test.

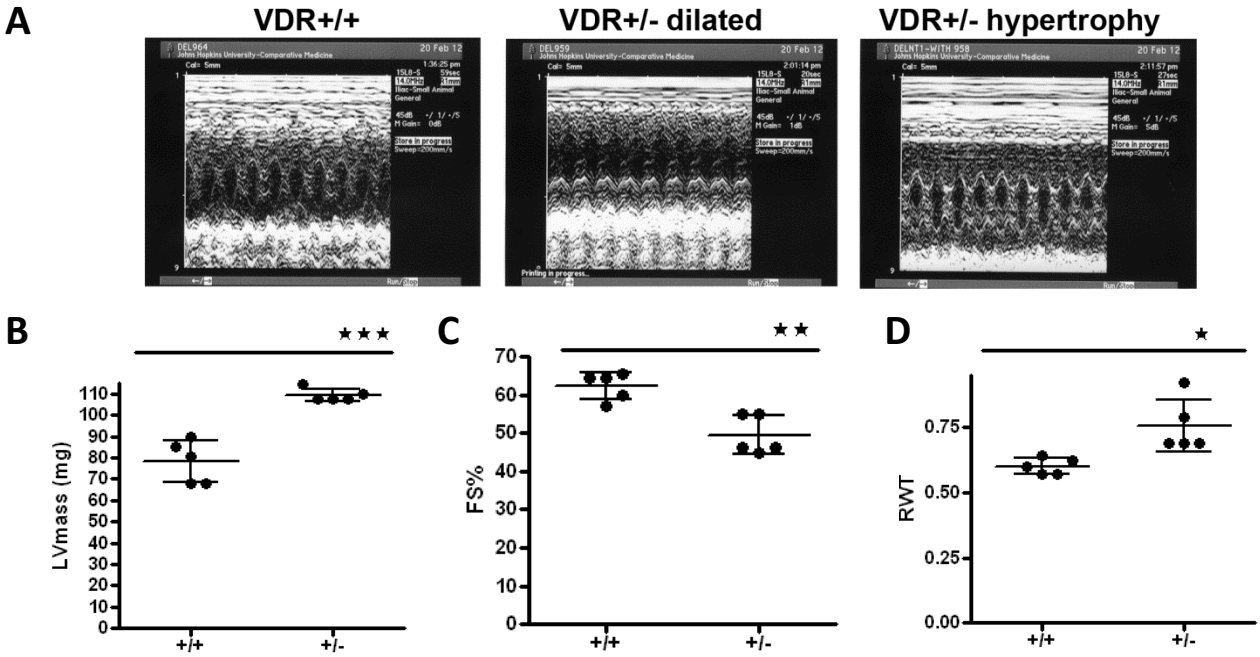


Figure 9. VDR+/- leads to hypertrophy and cardiac dysfunction in male mice with chronic CVB3 myocarditis. Male WT (VDR+/+), heterozygote (VDR+/-), and VDR -/- were infected ip with CVB3 on day 0 and echocardiography performed on day 35 pi on surviving mice. **A)** Short axis M-mode of LV in VDR+/+ and VDR+/- mice. Measurements taken from the echocardiogram included **B)** LV mass to assess hypertrophy, **C)** fractional shortening (%FS) to assess cardiac function, and **D)** relative wall thickness (RWT) to assess hypertrophy. Data show the mean +/-SEM comparing WT and VDR+/- groups using a 2-tailed Mann-Whitney rank sum test, *n*=5/ group.

VDR levels correlate to ejection fraction in sex specific manner in myocarditis patients

McNamara et al showed in 2011 that mainly men with myocarditis progress to DCM and heart failure (McNamara 2011). Based on our finding that VDR signaling appeared to increase myocarditis in males (**Fig 3**), we decided to examine whether a relationship existed between serum VitD levels in myocarditis patients and their %EF, as a marker of heart failure. A %EF of less than 45% is considered an indication of heart failure (Cooper 2009A,

McNamara 2011). The most common type of myocarditis found in the US is termed lymphocytic myocarditis (LM) (Cooper 2009A). Giant cell myocarditis (GCM) is a rare type of myocarditis that involves eosinophilic infiltration and the formation of macrophage accumulations called giant cells and typically rapidly progresses to dilation and heart failure (Cooper 2008, Cooper 2012). In this small pilot study, we were able to examine both of these myocarditis populations in collaboration with Dr. Leslie Cooper at Mayo Clinic.

In this pilot study, we examined serum VitD levels in a small number of lymphocytic myocarditis patients (*n*=16). We found that most patients had sufficient VitD levels (>20 ng/mL) (**Fig 10A**), and there was no relationship between sera VitD levels and poor cardiac function assessed as %EF when data from men and women were combined (**Fig 10B**). However, similar to the animal model, analysis by sex revealed that low VitD levels correlated with low/worse %EF in women with myocarditis, while higher VitD levels correlated with lower/worse %EF in men (**Fig 11**). This data, in a small sample of patients, suggest that few myocarditis patients have deficient levels of VitD and that there is no relationship to VitD and %EF. However, analysis by sex, shown in **Fig11**, below, reveals a different relationship between men and women.

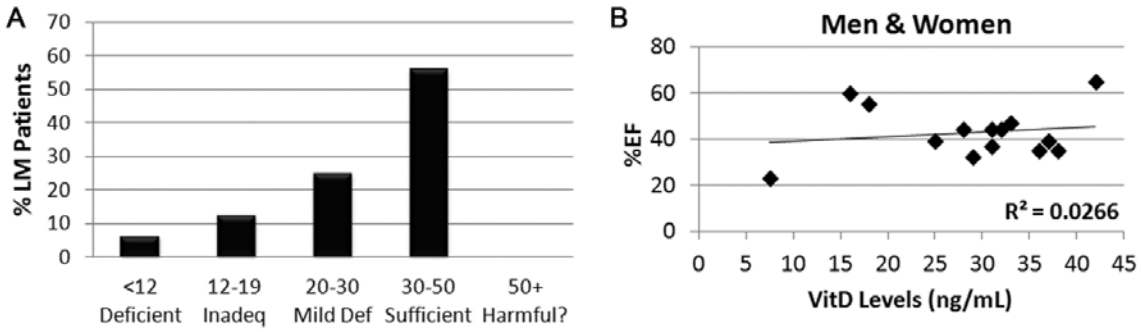


Figure 10. VitD levels in men and women with lymphocytic myocarditis in relation to cardiac function, in a small preliminary study. A) Percentage of men and women lymphocytic myocarditis

(LM) patients (same type of myocarditis as CVB3 mouse model) with deficient (<12 ng/mL), inadequate (Inadeq, 12-19 ng/mL), mildly deficient (Mild Def, 20-30 ng/mL), sufficient (30-50 ng/mL) or potentially harmful (50+ ng/mL) VitD levels in their sera assessed using LC-MS/MS (*n*=16). VitD category definitions based on the Institute of Medicine recommendations and the literature of commonly used categories (LaVie 2011, Ross 2011). **B)** Comparison of cardiac function based on %EF vs. serum VitD levels in myocarditis patients (R^2, 0.0266, *n*=16). Correlation between x and y values was calculated by liner regression and shown as R squared (R^2) values. R is defined as the correlation coefficient (R^2 values closer to 1 indicate high correlation between values)

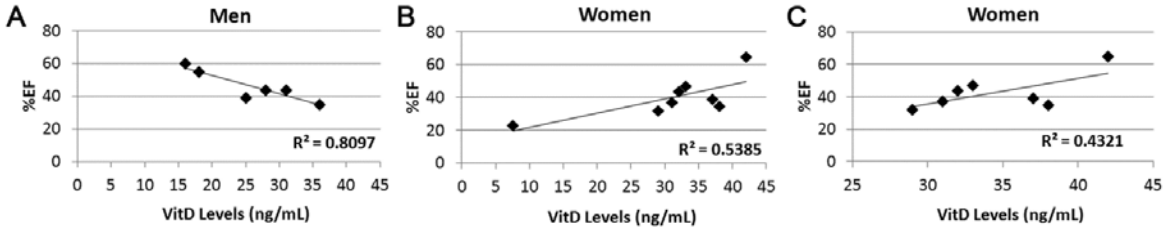


Figure 11. Correlation of VitD levels with cardiac function in LM patients according to sex. Because VitD is a sex steroid, VitD relationships should be analyzed according to sex. This type of analysis of the data in **Fig 10** reveals that worse cardiac function assessed as % ejection fraction (%EF) is associated with **A)** higher VitD levels in males (r^2=0.81, *n*=6) and **B&C)** lower VitD levels in women (r^2=0.54, *n*=10) with myocarditis. **C)** The correlation still exists in women if the lowest VitD level is removed (r^2=0.43) or the top value in removed (i.e., %EF 62, r^2=0.55, data not shown). Correlation between x and y values was calculated by liner regression and shown as R squared (R^2) values. R is defined as the correlation coefficient (R^2 values closer to 1 indicate high correlation between values).

When we examined a small number of GCM patients, we found that the majority of patients (75%) with GCM had low sera VitD levels (<20 ng/mL) when examining both sexes combined (**Fig 12A**). When the patient originally presented to the physician (when they were diagnosed and entered the study) we have termed "baseline", and sera from later dates (from

one month to two years after intial diagnosis) with the same patient after they received treatment for myocarditis/ heart failure is termed "treatment". We found no significant difference in sera VitD levels between men and women with myocarditis at Baseline (**Fig 12B**). However, we did find a significant difference in VitD levels in men and women with GCM after treatment (p=0.04) (**Fig 12B**). Since GCM has been found to be responsive to immunosuppressive treatments, these data suggest that VitD is playing a role in the inflammatory process in GCM in a sex-specific manner. These data, in a small sample of patients, suggest that low VitD is associated with GCM regardless of sex, and that women respond better than men to immunosuppressive treatment. The limitations of this study include the small numbers per group, all women in the study are post menopausal, and there is a wide range of follow-up dates for the patients in the "treatment" group.

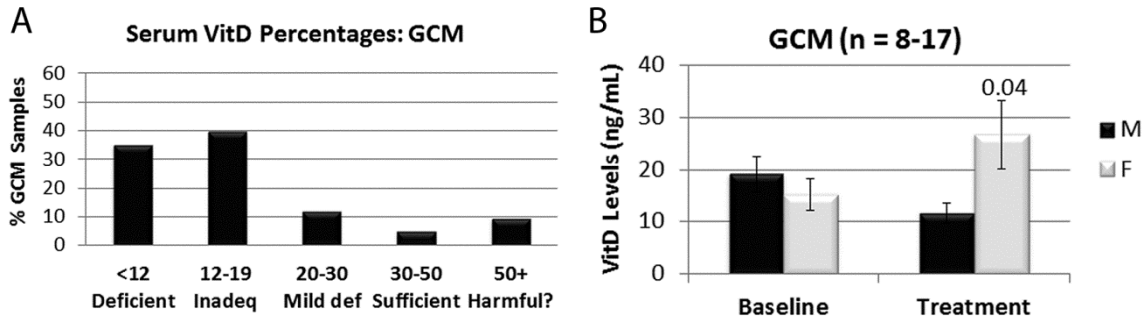


Figure 12. VitD levels in men and women with giant cell myocarditis before and after treatment in a small preliminary study. A) Percentage of men (M) and women (W) giant cell myocarditis (GCM) patients with deficient (<12ng/mL), inadequate (Inadeq, 12-19ng/mL), mildly deficient (Mild Def, 20-30ng/mL), sufficient (30-50ng/mL) or potentially harmful (50+ng/mL) VitD levels in their sera (n=16). Definitions based on (LaVie 2011, Ross 2011). **B)** Comparison of serum VitD levels in GCM (n=24) at time of diagnosis (Baseline) and after receiving treatment. Data show the mean +/- SEM of sera VitD levels by LC-MS/MS by a 2-tailed Mann-Whitney rank sum test, n=3-10/group.

Discussion

In this study we demonstrate that VDR signaling is important in driving sex differences in inflammation during CVB3 myocarditis in mice, with VDR protecting females and exacerbating disease in males. Using ovariectomy and hormone replacement we show that estrogen increases VDR expression in the heart of females, which may explain why WT females have more VDR expression in the heart than males prior to infection. This suggests that estrogen may be increasing VDR expression on cardiac resident cells rather than inflammatory cells. In contrast, increased expression of VDR in males with myocarditis suggests that VDR expression may be increased primarily in/on immune cells. Another possible explanation for the sex difference in VDR expression in the heart could be that male and female mice with myocarditis have different cell infiltrates with differing VDR expression. Males are likely to express VDR more on macropahges and mast cells, which are a greater proportion of the infiltrate in males with myocarditis (Frisancho-Kiss 2007). We did not find that testosterone increased VDR expression in the heart, but the analysis included the whole heart and we have found previously that testosterone only increased gene expression in CD11b+ immune cells rather than the whole heart (Fairweather 2014). Future studies will need to examine the effect of testosterone on gene expression by qRT-PCR using cells isolated from the heart or by flow cytometric analysis.

Interestingly, ERα, which is known to increase VDR expression, was significantly elevated in males with CVB3 myocarditis compared to control males (p=0.02, **Supplemental Table 1**), but not in females during myocarditis (**Supplemental Table 2**). Thus, ERα-mediated signaling may increase VDR expression during myocarditis in males, where estrogen acts as a growth/ survival factor for cardiac tissues (Buskiewicz 2015). Additionally,

the decrease in IL-1β in VDR-/- males indicates that VDR signaling increases this proinflammatory and profibrotic cytokine in males with myocarditis. Although we did not see a change in TLR4 and caspase-1 pathway expression in VDR-/- males with myocarditis compared to controls, these components are required for active IL-1β production and so must have been altered at an earlier timepoint. This will need to be confirmed in future experiments. These data suggest that VDR signaling contributes to elevated TLR4, caspase-1 and IL-1β in males with myocarditis.

Males may use VDR signaling in mast cells and macrophages as a strategy to eliminate CVB3 infection. Many studies have shown that VDR signaling activates innate immune pathways including the inflammasome to prevent viral and bacterial infections (Wang 2004, Liu 2009, Bikle 2011, Gambhir 2011, Hewison 2011, Lee 2011, Verway 2013, Wang 2010, Tulk 2015). Although in this context VDR signaling would be effective at eliminating CVB3 infection, it could also have the negative side effect of activating TLR2 and/or TLR4 and the inflammasome on/in mast cells, macrophages and cardiomyocytes. We showed previously that male BALB/c mice with CVB3 myocarditis have more mast cells and macrophages in the spleen and heart during the innate immune response and during acute myocarditis, respectively, as well as higher expression of TLR4 and other inflammasome-related genes (i.e., caspase-1, IL-1β, IL-18, IFNγ) than females (Fairweather 2003, Frisancho-Kiss 2007, Onyimba 2011, Coronado 2012, Fairweather 2014). We also previously demonstrated that testosterone increases an TLR4-mediated Th1-type immune response and this immunophenotype predisposes males to progress to DCM later (Frisancho-Kiss 2006B, Frisancho-Kiss 2009, Onyimba 2011, Coronado 2012, Fairweather 2014).

In contrast, females may use cytotoxic CD8 T cells, NK and/or natural killer T cells (NKT) cells instead of VDR signaling to eliminate CVB3, possibly via ERα signaling which is known to induce T, NK and NKT cell proliferation. Estrogen/ERα and VDR signaling are known to work together to increase T cell number and activation and to drive IL-4/Th2 and Treg responses- an immune environment that would not be effective at clearing virus (Buskiewicz 2015, Cantorna 2015, Hayes 2015, Piantoni 2015, Spanier 2015). Thus, activation of CD8+ T cells and/or NKT cells could be an effective viral control mechanism for females. Interestingly, VDR deficient females with myocarditis had significantly increased expression of T cell markers as well as the classic "male-type" immune profile with activation of TLR2/TLR4/IL-1R, caspase-1, and IL-1β, indicating that VDR signaling in females inhibits this pathway. Anti-viral strategies used by males and females appear to be sufficient, because there was no significant difference in cardiac viral replication according to sex during myocarditis.

Many studies report that VitD and/or VDR signialing increase IL-4/Th2 and Treg responses while inhibiting IFNγ/Th1 and IL-17/Th17 responses (Bikle 2011, Lisse 2011, Buskiewicz 2015, Cantorna 2015, Hayes 2015, Piantoni 2015, Spanier 2015, Toniato 2015, Trochoutsou 2015). However, most manuscripts and reviews of VitD/VDR effects on the immune response do not analyze or describe data by sex. Our data show that VDR signaling inhibits IFNγ/Th1/M1 and IL-17/Th17 responses only in female mice with CVB3 myocarditis. Based on the published literature and our findings here, VDR signaling could be responsible with testosterone for increasing TLR2, TLR4/IL-1R/caspase-1/IL-1β, IFNγ/Th1/M1, and IL-17A/Th17 proinflammatory responses in males. Our data suggest that

activation of [TLR4/caspase-1/]IL-1β, IFNγ/M1 macropahges, and mast cells (cKit) by VDR signaling in response to CVB3 infection elevates myocarditis in males.

There are a few scattered reports in the literature that suggest that sex differences in the inflammatory response to VitD and/or VDR exist. A small study compared healthy volunteer men and women and found that after two oral fat-loading tests VitD treatment reduced CD11b+ cells only in women (Klop 2014). In a separate study, women with low VitD levels were found to have more colorectal adenomas and women on VitD supplementation had lower numbers of adenomas, but this inverse association of VitD with adenoma number was not observed for men (Aigner 2014). A study in mice found that UVB radiation increased VitD levels in the skin of female, but not male mice (Xue 2015). This was found to be due to androgen receptor signaling, which lowered 7-dehydrocholesterol levels in the skin of males (Xue 2015). Two recent studies reported that women with low VitD levels were at an increased risk for myocardial infarction and this relationship was not observed for men (Karakas 2013, Verdoia 2015). Unfortunately, most animal and human studies of VitD/VDR do not study whether a sex difference in VitD levels and effects exist. Considering that all major CVDs are known to manifest sex differences in incidence, diagnosis, disease severity and/or progression combined with the epidemiologic data showing an increased risk for CVD based on VitD status, it is critically important to reanalyze past data and all future studies should specifically determine male and female responses. Adjusting for sex often prevents sex differences that exist in the data from being apparent. Our data in mice and the small study in patients suggest that women with low VitD levels may be at an increased risk of developing viral myocarditis, and that VDR activation in men could make disease more severe. Further studies with higher numbers are needed to

determine whether sex differences in VitD/VDR levels exist in myocarditis patients. Analyzing animal and human studies by sex will be critical to determine whether VitD supplementation or the use of VDR ligands (Crescioli 2014, Takada 2015) may benefit patients with myocarditis or other CVDs.

Supplementary Table 1. Vitamin D receptor response element (VDRE) gene changes during CVB3 myocarditis in the heart of males compared to undiseased males

GenBank	Gene	Gene name	Fold change	*p* value*
VDRE genes upregulated during myocarditis in males				
NM_008331	Ifit1	Interferon-induced protein with tetratricopeptide repeats 1	6.20	0.0002
NM_010501	Ifit3	Interferon-induced protein with tetratricopeptide repeats 3	5.99	0.0004
NM_021893	Cd274	CD274/ PD-L1	4.24	0.003
NM_008332	Ifit2	Interferon-induced protein with tetratricopeptide repeats 2	3.57	0.002
NM_010728	Lox	Lysyl oxidase	3.38	0.003
NM_009019	Rag1	Recombination activating gene 1	3.03	0.04
NM_172435	P2ry10	Purinergic receptor P2Y,G-protein coupled10	2.94	0.002
NM_021384	Rsad2	Radical S-adenosyl methionine domain containing 2	2.52	0.0008
NM_007609	Casp4	Caspase 4	2.48	0.0007
NM_007498	Atf3	Activating transcription factor 3	2.37	0.05
NM_001081211	Ptafr	Platelet-activating factor receptor	2.29	0.002
NM_009807	Casp1	Caspase 1 (interleukin 1beta convertase)	2.17	0.006
NM_0111898	Ptgs2	Prostaglandin-endoperoxide synthase 2	2.16	0.02

NM_009367	Tgfb2	Transforming growth factor, beta 2	2.11	0.03
NM_001127348	Snx10	Sorting nexin 10	2.10	0.002
NM_009256	Serpinb9	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 9	1.90	0.009
NM_001111021	Runx1	Runt-related transcription factor 1	1.90	0.01
NM_001033135	Rnf149	Ring finger protein 149	1.86	0.005
NM_00117646	Sirpa	Signal-regulatory protein alpha/ SHP-1	1.85	0.006
NM_001159394	Nfkbiz	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	1.85	0.01
NM_010496	Id2	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	1.81	0.001
NM_029023	Scpep1	Serine carboxypeptidase 1	1.80	0.002
NM_133662	Ier3	Immediate early response 3	1.78	0.01
NM_011932	Dapp1	Dual adaptor of phosphotyrosine and 3-phosphoinositides	1.77	0.008
NM_013589	Ltbp2	Latent transforming growth factor beta binding protein 2	1.76	0.03
NM_010720	Lipg	Lipase	1.74	0.006
NM_015759	Fgd3	FGD1 family, member 3	1.73	0.01
NM_030710	Slamf6	Kallmann syndrome 1 sequence	1.72	0.004
NM_008062	G6pdx	Glucose-6-phosphate dehydrogenase x-linked	1.71	0.004
NM_022019	Dusp10	Dual specificity phosphatase 10	1.70	0.006

NM_019388	Cd86	CD86 antigen (B7-2 antigen)	1.68	0.04
NM_009655	Alcam	Activated leukocyte cell adhesion molecule	1.68	0.0007
NM_008390	Irf1	Interferon regulatory factor 1	1.67	0.009
NM_011201	Ptpn1	Protein tyrosine phosphatase, non-receptor 1	1.66	0.008
NM_019494	Cxcl11	Chemokine (C-X-C motif) ligand 11	1.66	0.04
NM_007742	Col1a1	Collagen, type I, alpha 1	1.66	0.02
NM_001136079	Ptger4	Prostaglandin E receptor 4 (subtype EP4)	1.65	0.002
NM_134164	Syt12	Synaptotagmin XII	1.64	0.009
NM_001163540	Plec1	Plectin 1	1.64	0.002
NM_007730	Col12a1	Collagen, type XII, alpha 1	1.63	0.02
NM_001111096	Lyn	V-yes-1 Yamaguchi sarcoma viral related oncogene homolog	1.62	0.02
NM_009264	Sprr1a	Small proline-rich protein 1A	1.60	0.0009
NM_027677	Gpr39	G protein-coupled receptor 39	1.59	0.008
NM_001009947	Dock11	Dedicator of cytokinesis 11	1.58	0.01
NM_001145960	Slc37a2	Solute carrier family 37 (glycerol-3-phosphate transporter), member 2	1.57	0.02
NM_009841	Cd14	CD14 antigen	1.55	0.04
NM_016911	Srpx	Sushi-repeat-containing protein, X-linked	1.53	0.009
NM_008494	Lfng	Lunatic fringe homolog	1.53	0.003
NM_181402	Parp11	Poly (ADP-ribose) polymerase family 11	1.51	0.02

NM_001037717	Slc38a6	Solute carrier family 38, member 6	1.49	0.001
NM_031167	Il1rn	Interleukin 1 receptor antagonist	1.47	0.02
NM_010658	Mafb	V-maf musculoaponeurotic fibrosarcoma oncogene homolog B	1.47	0.02
NM_001198914	Myb	V-myb myeloblastosis viral oncogene homolog	1.46	0.03
NM_175188	March	Membrane-associated RING-CH protein I	1.42	0.02
NM_008129	Gclm	Glutamate-cysteine ligase, modifier subunit	1.42	0.002
NG_003057	Serpinb 1a	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 1	1.42	0.02
NM_053109	Clec2d	C-type lectin superfamily 2, member D	1.41	0.002
NM_008047	Fstl1	Follistatin-like 1	1.41	0.03
NM_028421	Zc3hav1	Zinc finger CCCH type, antiviral 1	1.39	0.02
NM_007464	Birc3	Baculoviral IAP repeat-containing 3	1.39	0.02
NM_001135727	Sh3kbp1	SH3-domain kinase binding protein 1	1.37	0.002
NM_013650	S100a8	S100 calcium binding protein A8	1.37	0.04
NM_009255	Serpine2	Serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	1.36	0.03
NM_009302	Swap70	SWAP-70 protein	1.36	0.004
NM_013673	Sp100	Nuclear antigen Sp100	1.36	0.03

NM_153139	Slc36a1	Solute carrier family 36 (proton/amino acid symporter), member 1	1.33	0.01
NM_011479	Sptlc2	Serine palmitoyltransferase, subunit 2	1.33	0.02
NM_001080813	Rab11fip1	RAB11 family interacting protein 1 (class I)	1.33	0.03
NM_173370	Cds1	CDP-diacylglycerol synthase 1	1.33	0.01
NM_007746	Map3k8	Mitogen-activated protein 3x-kinase 8	1.32	0.04
NM_010941	Nsdhl	NAD(P)-dep steroid dehydrogenase-like	1.31	0.001
NM_001008700	Il4ra	Interleukin 4 receptor alpha	1.30	0.09
NM_138952	Ripk2	receptor-interacting serine-threonine kinase 2	1.30	0.002
NM_172779	Ddx26b	DEAD/H box polypeptide 26B	1.29	0.004
NM_026678	Blvra	Biliverdin reductase A	1.28	0.02
NM_001037177	Nfatc2	Nuclear factor of activated T-cells 2	1.27	0.01
NM_009114	S100a9	S100 calcium binding protein A9	1.26	0.04
NM_001081011	Srgap2	SLIT-ROBO Rho GTPase activating protein 2	1.26	0.01
NM_001080548	Usp6nl	USP6 N-terminal like	1.25	0.04
NM_013454	Abca1	ATP-binding cassette, sub-family A (ABC1)	1.24	0.02
NM_010809	Mmp3	Matrix metalloproteinase 3	1.24	0.01
NM_007956	Esr1	Estrogen receptor 1	1.24	0.02
NM_024239	Stambp	STAM binding protein	1.24	0.005

NM_011200	Ptp4a1	Protein tyrosine phosphatase type IVA, 1	1.24	0.006
NM_007658	Cdc25a	Cell division cycle 25A	1.24	0.004
NM_172702	Serinc2	Tumor differentially expressed 2-like	1.22	0.03
NM_026281	Tm7sf3	Transmembrane 7 superfamily member 3	1.21	0.003
NM_028523	Dcbld2	Discoidin, CUB and LCCL domain containing	1.21	0.04
NM_008179	Gspt2	G1 to S phase transition 2	1.19	0.03
NM_018886	Lgals8	Galectin 8	1.18	0.03
NM_001042513	Txnrd1	Thioredoxin reductase 1	1.18	0.02
NM_001164604	Nsmce2	Breast cancer membrane protein 101	1.17	0.04
NM_009504	Vdr	Vitamin D receptor	1.16	0.04
NM_177586	Eif5a2	Eukaryotic translation initiation factor 5A2	1.16	0.02
NM_172288	Nup133	Nucleoporin 133kDa	1.15	0.03
NM_011897	Spry2	Sprouty homolog 2	1.15	0.03
NM_133838	Ehd4	EH-domain containing 4	1.13	0.04
NM_024195	Cyb5r4	NADPH cytochrome B5 oxidoreductase	1.12	0.01
VDRE genes downregulated during myocarditis in males				
NM_001081276	Clasp1	Cytoplasmic linker associated protein 1	-1.59	1.7E-5
NM_022982	Rtn4r	Reticulon 4 receptor	-1.56	0.04
NM_14926	Sez6l2	Seizure related 6 homolog-like 2	-1.52	0.002
NM_026346	Fbxo32	F-box protein 32	-1.49	0.03

NM_008882	Plxna2	Plexin A2	-1.49	0.0009
NM_133754	Fblim1	Filamin-binding LIM protein-1	-1.46	0.004
NM_017391	Slc5a3	Solute carrier family 5, member 3	-1.44	0.009
NM_026880	Pink1	PTEN-induced putative kinase 1	-1.42	0.002
NM_007382	Acadm	Acyl-coenzyme A dehydrogenase	-1.42	0.004
NM_007425	Ager	Renal tumor antigen/ RAGE	-1.41	0.004
NM_001170978	Abat	4-aminobutyrate aminotransferase	-1.41	0.01
NM_008650	Mut	Methylmalonyl coenzyme A mutase	-1.40	0.01
NM_021715	Chst7	Carbohydrate sulfotransferase 7	-1.40	0.004
NM_001122759	Pde7a	Phosphodiesterase 7A	-1.39	0.01
NM_007416	Adra1b	Adrenergic receptor alpha-1b	-1.38	0.02
NM_001013777	Zfp488	Zinc finger protein 488	-1.37	0.03
NM_007445	Amh	Anti-Mullerian hormone	-1.36	4.0E-5
NM_007731	Col13a1	Collagen, type XIII, alpha 1	-1.36	0.009
NM_001040682	Clmn	Calmin (calponin-like, transmembrane)	-1.35	0.04
NM_010637	Klf4	Kruppel-like factor 4 (gut)	-1.35	0.0002
NM_031185	Akap12	A kinase (PRKA) anchor protein 12	-1.35	0.007
NM_001243132	Tspan2	Tetraspan 2	-1.34	0.01
NM_172532	Aldh5a1	Aldehyde dehydrogenase 5 family, A1	-1.34	0.004
NM_175443	Etnk2	Ethanolamine kinase 2	-1.33	0.001
NM_010097	Sparcl1	SPARC-like 1 (mast9, hevin)	-1.32	0.0001
NM_172420	Ppp1r1c	Protein phosphatase 1, regulatory 1C	-1.32	0.005
NM_001025250	Vegfa	Vascular endothelial growth factor	-1.31	0.04

NM_001103177	Ablim1	Actin binding LIM protein 1	-1.31	4.9E-5
NM_138956	Rassf3	Ras association domain family 3	-1.30	0.0006
NM_001164572	Snrk	SNF-1 related kinase	-1.30	0.02
NM_025862	Acad8	Acyl-coenzyme A dehydrogenase 8	-1.30	0.003
NM_019739	Foxo1	Forkhead box O1A	-1.29	0.04
NM_001081977	Rnf144a	Ring finger protein 144	-1.29	0.01
NM_008984	Ptprm	Protein tyrosine phosphatase, M	-1.29	0.02
NM_010111	Efnb2	Ephrin-B2	-1.28	0.02
NM_001039509	Pnkd	Myofibrillogenesis regulator 1	-1.28	0.02
NM_009662	Alox5	Arachidonate 5-lipoxygenase	-1.28	0.04
NM_026385	Pllp	Plasmolipin	-1.27	0.007
NM_008090	Gata2	GATA binding protein 2	-1.27	0.01
NM_029653	Dapk1	Death-associated protein kinase 1	-1.27	0.04
NM_178357	Klf11	Kruppel-like factor 11	-1.26	0.04
NM_001166506	Sec14l1	SEC14-like 1	-1.26	0.009
NM_001252341	Syt1	Synaptotagmin I	-1.26	0.01
NM_010340	Gpr50	G protein-coupled receptor 50	-1.26	0.03
NM_001164717	Sh3pxd2a	SH3 multiple domains 1	-1.25	0.008
NM_010778	Cd46	Membrane cofactor protein (CD46)	-1.24	0.04
NM_172671	Lgr4	Leucine-rich repeat-containing G protein-coupled receptor 4	-1.24	0.04
NM_001177849	Asph	Aspartate beta-hydroxylase	-1.24	8.9E-5
NM_022413	Trpv6	Transient receptor potential cation	-1.24	0.04

		channel V6		
NM_010570	Irs1	Insulin receptor substrate 1	-1.23	0.02
NM_021883	Tmod1	Tropomodulin 1	-1.23	0.02
NM_001077403	Nrp2	Neuropilin 2	-1.23	0.04
NM_010588	Jag2	Jagged 2	-1.22	0.0004
NM_012026	Rgnef	Rho-guanine nucleotide exchange factor	-1.22	0.02
NM_010119	Ehd1	EH-domain containing 1	-1.22	0.04
NM_019563	Cited4	Cbp/p300-interacting transactivator 4	-1.21	0.02
NM_177839	Tnn	Tenascin N	-1.20	0.02
NM_001163512	Rgs12	Regulator of G-protein signalling 12	-1.20	0.009
NM_001008548	Pde2a	Phosphodiesterase 2A, cGMP-stimulated	-1.20	0.04
NM_011177	Klk6	Kallikrein 6 (neurosin, zyme)	-1.20	0.008
NM_053207	Egln1	Egl nine homolog 1	-1.19	0.003
MN_001005863	Mtus1	Mitochondrial tumor suppressor 1	-1.19	0.04
NM_207666	Dlk2	EGF-like-domain/ delta-like 2 homolog	-1.19	0.04
NM 030067	Gpr115	G protein-coupled receptor 115	-1.18	0.003
NM_001111272	Stau2	Staufen, RNA binding protein, homolog 2	-1.18	5.4E-5
NM_027174	Col22a1	Collagen, type XXII, alpha 1	-1.17	0.04
NM_018784	St3gal6	ST3 β-galactoside α-2,3-sialyltransferase 6	-1.17	0.0003

NM_001199151	Sncaip	Synuclein, alpha interacting protein (synphilin)	-1.17	0.03
NM_001172424	Dhrs3	Dehydrogenase/reductase member 3	-1.17	0.04
NM_152915	Dner	Delta-notch-like EGF repeat-containing	-1.17	0.04
NM_008290	Hsd17b2	Hydroxysteroid (17-beta) dehydrogenase 2	-1.16	0.03
NM_008861	Pkd2	Polycystic kidney disease 2	-1.15	0.02
NM_011170	Prnp	Prion protein/ Creutzfeld-Jakob disease	-1.14	0.01
NM_172266	Lpgat1	Lysophosphatidylglycerol acyltransferase 1	-1.14	0.002
NM_001081960	Clasp2	Cytoplasmic linker associated protein 2	-1.13	0.04
NM_001130109	Ptk2	PTK2 protein tyrosine kinase 2	-1.13	0.04
NM_001029850	Magi1	Membrane associated guanylate kinase interacting protein-like 1	-1.13	0.04
NM_001001602	Dab2ip	DAB2 interacting protein	-1.12	0.04
NM_029466	Arl5b	ADP-ribosylation factor-like 8	-1.12	0.03
NM_010434	Hipk3	Homeodomain interacting protein kinase 3	-1.11	0.04
NM_001146059	Als2cl	ALS2 C-terminal like	-1.11	0.04
NM_001135559	Sos2	Son of sevenless homolog 2	-1.09	0.02
NM_212433	Fbxo3	F-box protein 3	-1.08	0.04
NM_009579	Slc30a1	Solute carrier family 30 (zinc transporter), member 1	-1.07	0.03

NM_009303	Syngr1	Synaptogyrin 1	-1.07	0.01
NM_010629	Kifap3	Kinesin-associated protein 3	-1.07	0.03

*p values compare the heart of males with myocarditis to undiseased males at day 10 pi

Supplementary Table 2. Vitamin D receptor response element (VDRE) gene changes during CVB3 myocarditis in the heart of females compared to undiseased females

GenBank	Gene	Gene name	Fold change	*p* value*
VDRE genes upregulated during myocarditis in females				
NM_021893	Cd274	CD274/ programmed cell death 1 ligand 1 (PD-L1)	4.03	0.003
NM_007609	Casp4	caspase 4, apoptosis-related cysteine protease	2.16	0.002
NM_021384	Rsad2	radical S-adenosyl methionine domain containing 2	2.12	0.003
NM_019494	Cxcl11	chemokine (C-X-C motif) ligand 11	2.03	0.01
NM_001111021	Runx1	runt-related transcription factor 1 (acute myeloid leukemia 1; aml1 oncogene)	1.91	0.01
NM_172435	P2ry10	purinergic receptor P2Y, G-protein coupled10	1.91	0.02
NM_001081211	Ptafr	platelet-activating factor receptor	1.82	0.01
NM_001159394	Nfkbiz	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	1.80	0.02
NM_019388	Cd86	CD86 antigen (CD28 antigen ligand 2, B7-2 antigen)	1.77	0.03
NM_009807	Casp1	caspase 1, apoptosis-related cysteine protease (interleukin 1, beta, convertase)	1.75	0.03

NM_00117646	Sirpa	signal-regulatory protein alpha/ SHP-1	1.64	0.02
NM_009256	Serpinb9	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 9	1.63	0.03
NM_181402	Parp11	poly (ADP-ribose) polymerase family, member 11	1.52	0.02
NM_001033135	Rnf149	ring finger protein 149	1.50	0.03
NM_001163540	Plec1	plectin 1, intermediate filament binding protein 500kDa	1.50	0.006
NM_001127348	Snx10	sorting nexin 10	1.49	0.04
NM_029023	Scpep1	serine carboxypeptidase 1	1.43	0.02
NM_013673	Sp100	Nuclear antigen Sp100	1.38	0.02
NM_028421	Zc3hav1	zinc finger CCCH type, antiviral 1	1.35	0.03
NM_001037717	Slc38a6	solute carrier family 38, member 6	1.26	0.03
NM_001145920	Runx2	Runt-related transcription factor 2	1.21	0.01
NM_001135727	Sh3kbp1	SH3-domain kinase binding protein 1	1.21	0.03
NM_053109	Clec2d	C-type lectin superfamily 2, member D	1.20	0.04
NM_001079883	Bcl11b	B-cell CLL/lymphoma 11B (zinc finger protein)	1.21	0.04
NM_011188	Psmc2	Proteasome (prosome, macropain) 26S subunit, ATPase, 2	1.19	0.02
NM_011374	St8sia1	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1	1.19	0.0004
NM_019689	Arid3b	AT rich interactive domain 3B	1.12	0.02

VDRE genes downregulated during myocarditis in females

NM_183216	Scd4	stearoyl-CoA desaturase 4	-2.16	0.009
NM_001024955	Pik3r1	phosphoinositide-3-kinase, regulatory subunit	-1.45	0.03
NM_001170978	Abat	4-Aminobutyrate aminotransferase	-1.41	0.01
NM_026880	Pink1	PTEN induced putative kinase 1	-1.39	0.003
	Dgat2	diacylglycerol O-acyltransferase homolog 2 (mouse)	-1.39	0.05
NM_153587	Rps6ka5	ribosomal protein S6 kinase, 90kDa, polypeptide 5	-1.39	0.02
	Wnt5a	wingless-type MMTV integration site family, member 5A	-1.36	0.03
NM_172532	Aldh5a1	aldehyde dehydrogenase 5 family, member A1 (succinate-semialdehyde dehydrogenase)	-1.36	0.003
NM_008650	Mut	methylmalonyl Coenzyme A mutase	-1.33	0.02
NM_001008548	Pde2a	phosphodiesterase 2A, cGMP-stimulated	-1.30	0.01
NM_001252341	Syt1	synaptotagmin I	-1.28	0.006
NM_008882	Plxna2	plexin A2	-1.28	0.01
NM_017391	Slc5a3	solute carrier family 5 (inositol transporters), member 3	-1.28	0.04
NM_007382	Acadm	acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain	-1.28	0.02

NM_001122758	Pcdh7	BH-protocadherin (brain-heart)	-1.28	0.002
NM_007556	Bmp6	bone morphogenetic protein 6	-1.27	0.04
	Tceal1	transcription elongation factor A (SII)-like 1	-1.27	0.01
NM_023270	Rnf128	ring finger protein 128	-1.26	0.008
NM_001146059	Als2cl	ALS2 C-terminal like	-1.26	0.001
NM_009662	Alox5	arachidonate 5-lipoxygenase	-1.25	0.05
NM_001164034	Ntf3	neurotrophin 3	-1.24	0.04
NM_001081146	Prickle2	prickle-like 2 (Drosophila)	-1.22	0.01
NM_021883	Tmod1	tropomodulin 1	-1.21	0.02
NM_010119	Ehd1	EH-domain containing 1	-1.21	0.05
	Ulk1	unc-51-like kinase 1 (C. elegans)	-1.20	0.02
NM_021439	Chst11	carbohydrate (chondroitin 4) sulfotransferase 1	-1.20	0.02
NM_025862	Acad8	acyl-Coenzyme A dehydrogenase family, member 8	-1.20	0.02
NM_013723	Podxl	podocalyxin-like	-1.19	0.02
NM_018784	St3gal6	ST3 beta-galactoside alpha-2,3-sialyltransferase 6	-1.19	0.0001
NM_008984	Ptprm	protein tyrosine phosphatase, receptor type, M	-1.18	0.08
NM_011897	Spry2	sprouty homolog 2 (Drosophila)	-1.18	0.01
NM_001038621	Rabgap1l	RAB GTPase activating protein 1-like	-1.17	0.04

NM_138956	Rassf3	Ras association (RalGDS/AF-6) domain family 3	-1.17	0.01
NM_053207	Egln1	egl nine homolog 1 (C. elegans)	-1.167	0.005
NM_194344	Sh3tc1	SH3 domain and tetratricopeptide repeats 1	-1.17	0.006
NM_001103177	Ablim1	actin binding LIM protein 1	-1.17	0.002
NM_001001602	Dab2ip	DAB2 interacting protein	-1.15	0.02
NM_011804	Creg1	cellular repressor of E1A-stimulated genes 1 (females)	-1.15	0.02
NM_001177849	Asph	aspartate beta-hydroxylase (cardiac remodeling)	-1.14	0.003
NM_0.0179	Clip4	restin-like 2	-1.13	0.002
NM_008783	Pbx1	Pre-B-cell leukemia transcription factor 1 (females)	-1.12	0.02
NM_008970	Pthlh	Parathyroid hormone-like hormone	-1.12	0.04
NM_010097	Sparcl1	SPARC-like 1 (mast9, hevin)	-1.12	0.02
NM_153178	Eif2c2	Eukaryotic translation initiation factor 2C, 2	-1.11	0.04
NM_001111272	Stau2	staufen, RNA binding protein, homolog 2 (Drosophila)	-1.11	0.001
	Slc25a4	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4	-1.10	0.03

	Trip11	thyroid hormone receptor interactor 11	-1.10	0.03
	Pcnx	pecanex homolog (Drosophila)	-1.09	0.05
NM_009579	Slc30a1	Solute carrier family 30 (zinc transporter), member 1	-1.09	0.01
NM_025446	Aig1	Androgen-induced 1	-1.09	0.04
	Capn7	calpain 7 (?females)	-1.08	0.05
NM_009303	Syngr1	synaptogyrin 1	-1.07	0.008
	Ppp3ca	Protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform (calcineurin A alpha)	-1.07	0.01
NM_001025250	Vegfa	vascular endothelial growth factor	-1.03	8.3E-1

**p* values compare the heart of females with myocarditis to undiseased females at day 10 pi

Supplementary Table 3. Vitamin D receptor response element (VDRE) gene changes in the heart of undiseased males compared to undiseased females

GenBank	Gene	Gene name	Fold change	*p* value*
VDRE genes upregulated in undiseased males compared to undiseased females				
NM_019568	Cxcl14	chemokine (C-X-C motif) ligand 14	1.81	0.007
NM_009616	Adam19	A disintegrin and metalloproteinase domain 19 (meltrin beta)	1.43	0.04
NM_007425	Ager	renal tumor antigen	1.36	0.008
NM_009255	Serpine2	Serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	1.33	0.04
NM_001112698	Ngf	nerve growth factor, beta polypeptide	1.27	0.06
NM_009446	Tuba3a	tubulin, alpha 3	1.26	0.04
NM_007731	Col13a1	collagen, type XIII, alpha 1	1.25	0.04
NM_026385	Pllp	transmembrane 4 superfamily member 11 (plasmolipin)	1.24	0.01
NM_001079883	Bcl11b	B-cell CLL/lymphoma 11B (zinc finger protein)	1.22	0.03
NM_011170	Prnp	prion protein (p27-30) (Creutzfeld-Jakob disease, Gerstmann-Strausler-Scheinker syndrome, fatal familial insomnia)	1.22	0.0009
NM_011349	Sema3f	sema domain, immunoglobulin domain	1.21	0.01

		(Ig), short basic domain, secreted, (semaphorin) 3F		
NM_029961	Abcb5	ATP-binding cassette, sub-family B (MDR/TAP), member 5	1.17	0.06
NM_009199	Slc1a1	solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1	1.17	0.03
NM_001195083	Phc2	Polyhomeotic-like 2 (Drosophila)	1.16	0.04
NM_001013616	Trim6	tripartite motif-containing 6	1.14	0.07
NM_025821	Carhsp1	calcium regulated heat stable protein 1, 24kDa	1.13	0.07
NM_199465	Nexn	nexilin (F actin binding protein)	1.07	0.03
VDRE genes downregulated in undiseased males compared to undiseased females				
NM_022019	Dusp10	dual specificity phosphatase 10	-1.47	0.03
NM_153587	Rps6ka5	ribosomal protein S6 kinase, 90kDa, polypeptide 5	-1.37	0.03
NM_001177713	Cyp26b1	cytochrome P450, family 26, subfamily B, polypeptide 1	-1.34	0.03
NM_027629	Pgm2l1	phosphoglucomutase 2-like 1	-1.30	0.009
NM_007499	Atm	ataxia telangiectasia mutated (includes complementation groups A, C and D)	-1.31	0.01

NM_146236	Tceal1	transcription elongation factor A (SII)-like 1	-1.25	0.02
NM_010049	Dhfr	dihydrofolate reductase	-1.25	0.02
NM_001122758	Pcdh7	BH-protocadherin (brain-heart)	-1.24	0.004
NM_001113351	Synj2	synaptojanin 2	-1.24	0.04
NM_031402	Crispld1	LCCL domain containing cysteine-rich secretory protein 1	-1.21	0.02
NM_007440	Alox12	arachidonate 12-lipoxygenase	-1.21	0.06
NM_172288	Nup133	Nucleoporin 133kDa	-1.20	0.009
NM_172779	Ddx26b	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 26B	-1.19	0.03
NM_177224	Chd9	Chromodomain helicase DNA binding protein 9	-1.19	0.03
NM_001164604	Nsmce2	breast cancer membrane protein 101	-1.18	0.03
NM_138952	Ripk2	receptor-interacting serine-threonine kinase 2	-1.17	0.03
NM_001038621	Rabgap1l	RAB GTPase activating protein 1-like	-1.16	0.05
NM_007658	Cdc25a	cell division cycle 25A	-1.16	0.03
NM_025347	Ypel3	yippee-like 3 (Drosophila)	-1.15	0.05
NM_011594	Timp2	tissue inhibitor of metalloproteinase 2	-1.15	0.009
NM_026281	Tm7sf3	Transmembrane 7 superfamily member 3	-1.14	0.02
NM_212446	Psmf1	Proteasome (prosome, macropain) inhibitor subunit 1 (PI31)	-1.11	0.04

NM_009796	Capn7	calpain 7	-1.07	0.05

*p values compare undiseased male hearts with undiseased female hearts at day 10 pi

Supplementary Table 4. Vitamin D receptor response element (VDRE) gene changes in the heart of males vs. females with acute CVB3 myocarditis

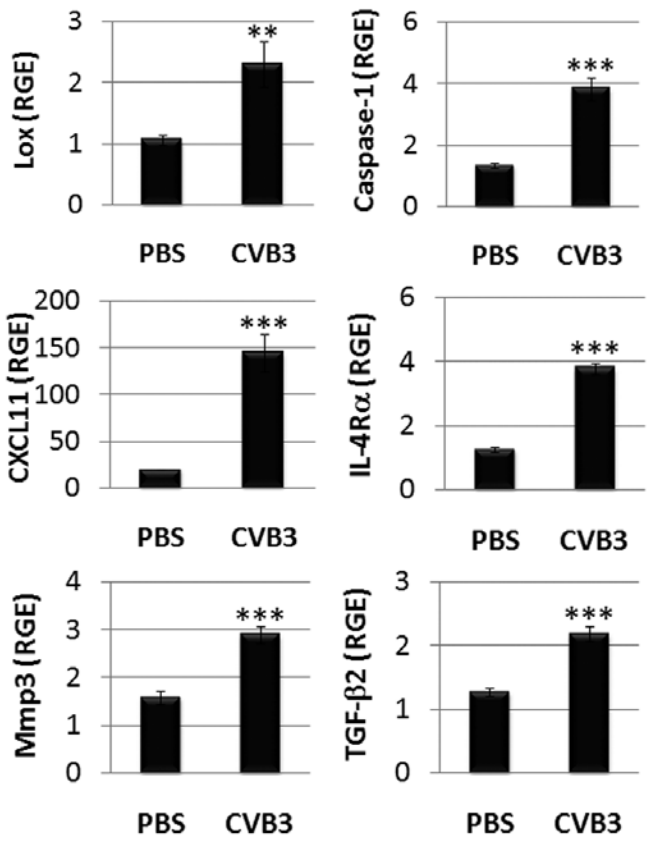
GenBank	Gene	Gene name	Fold change	*p* value*
VDRE genes upregulated in males during myocarditis				
NM_009019	Rag1	recombination activating gene 1	3.32	0.03
NM_019568	Cxcl14	chemokine (C-X-C motif) ligand 14	1.86	0.005
NM_0111898	Ptgs2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	1.80	0.06
NM_016911	Srpx	sushi-repeat-containing protein, X-linked	1.67	0.003
NM_134164	Syt12	synaptotagmin XII	1.60	0.01
NM_019662	Serpine2	Serine (or cysteine) proteinase inhibitor, clade E (nexin), plasminogen activator inhibitor type 1), member 2	1.57	0.006
NM_001198914	Rrad	Ras-related associated with diabetes	1.57	0.02
NM_009264	Myb	v-myb myeloblastosis viral oncogene homolog (avian)	1.42	0.05
NM_021361	Col12a1	Collagen, type XII, alpha 1	1.41	0.07
NM_008542	S100a8	S100 calcium binding protein A8 (calgranulin A)	1.40	0.03
NM_009114	P2ry2	purinergic receptor P2Y, G-protein coupled, 2	1.40	0.009

NM_029961	Ptger4	prostaglandin E receptor 4 (subtype EP4)	1.39	0.02
NM_009423	Sprr1a	small proline-rich protein 1A	1.39	0.007
NM_001130476	Nova1	neuro-oncological ventral antigen 1	1.30	0.002
NM_011200	Smad6	SMAD, mothers against DPP homolog 6 (Drosophila)	1.29	0.04
NM_133252	S100a9	S100 calcium binding protein A9	1.26	0.05
NM_025821	Carhsp1	calcium regulated heat stable protein 1, 24kDa	1.19	0.02
NM_001042513	Txnrd1	thioredoxin reductase 1	1.16	0.02
NM_153178	Eif2c2	Eukaryotic translation initiation factor 2C, 2	1.16	0.008
NM_029582	Txndc11	thioredoxin domain containing 11	1.16	0.02
NM_013659	Sema4b	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4B	1.16	0.02
NM_011897	Spry2	sprouty homolog 2 (Drosophila)	1.14	0.03
NM_194344	Sh3tc1	SH3 domain and tetratricopeptide repeats 1	1.14	0.01
NM_011170	Prnp	prion protein (p27-30) (Creutzfeld-Jakob disease, Gerstmann-Strausler-Scheinker syndrome, fatal familial insomnia)	1.13	0.01
NM_054078	Baz2a	Bromodomain adjacent to zinc finger	1.12	0.02

		domain, 2A		
NM_026281	Tm7sf3	Transmembrane 7 superfamily member 3	1.11	0.05
VDRE genes downregulated in males during myocarditis				
NM_14926	Sez6I2	seizure related 6 homolog (mouse)-like 2	-1.47	0.003
NM_001122759	Pde7a	Phosphodiesterase 7A	-1.42	0.009
NM_028711	Slc25a27	solute carrier family 25, member 27	-1.32	0.02
NM_001081276	Clasp1	cytoplasmic linker associated protein 1	-1.31	0.0007
NM_009200	Slc1a6	solute carrier family 1 (high affinity aspartate/glutamate transporter), member 6	-1.27	0.02
NM_007445	Amh	anti-Mullerian hormone	-1.25	0.0003
NM_031185	Akap12	A kinase (PRKA) anchor protein 12	-1.24	0.03
NM_010097	Sparcl1	SPARC-like 1 (mast9, hevin)	-1.24	0.0007
NM_021715	Chst7	carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 7	-1.21	0.06
NM_007440	Alox12	arachidonate 12-lipoxygenase	-1.20	0.05
NM_172420	Ppp1r1c	protein phosphatase 1, regulatory (inhibitor) subunit 1C	-1.20	0.04
NM_028307	Tdrkh	tudor and KH domain containing	-1.20	0.01
NM_011374	St8sia1	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1	-1.19	0.0005
NM_001081977	Rnf144a	ring finger protein 144	-1.18	0.08
NM_207666	Dlk2	EGF-like-domain/ delta-like 2 homolog	-1.18	0.06

NM_177580	Baiap2l2	BAI1-associated protein 2-like 2	-1.17	0.04
NM_177839	Tnn	tenascin N	-1.16	0.05
NM_020626	Tmem27	transmembrane protein 27	-1.10	0.03
NM_001177849	Asph	aspartate beta-hydroxylase (cardiac remodeling)	-1.10	0.01

**p* values compare the hearts of males vs. female during myocarditis at day 10 pi



Supplementary Figure 1. Verification of microarray results of VDRE genes upregulated during myocarditis in males. WT mice were infected ip with CVB3 on day 0 and mRNA expression was examined in whole hearts at day 10 pi by qRT-PCR and compared to uninfected male mice to confirm VDRE pro-inflammatory (COX, Caspase-1, CXCL11), regulatory (IL-4Rα and TGFβ2) and profibrotic (TGFβ2 and Mmp3) genes found by microarray data. Data show the mean +/-SEM relative gene expression (RGE) compared to HPRT by a 2-tailed Mann-Whitney rank sum test, *n*=3-10/group.

Chapter 4

BPA increases myocarditis in female mice in plastic cages by increasing mast cell/inflammasome/Th1 responses and by altering estrogen and vitamin D receptors

Abstract

Myocarditis is an inflammatory heart disease that leads to DCM and heart failure. Sex hormones play a vital role in development of myocarditis with testosterone driving disease in males. Whereas, estrogen, via ERα, mediates cardioprotection in females. Since myocarditis is influenced by sex hormones, it is highly probable that endocrine disruptors, which interfere with natural hormones, will play a part in the progression of the disease. The human population is widely exposed to the endocrine disruptor BPA from plastics, such as water bottles, plastic food containers, copy paper, and receipts. BPA has been found to act through ERβ in cardiomyocytes to increase cardiac arrhythmias. Thus, BPA could increase myocarditis through deleterious actions of the ERβ rather than beneficial effects via ERα. We also found that VDR signaling is protective in females during myocarditis. To our knowledge no one has examined the role of endocrine disruptors like BPA on myocarditis. We found that a human relevant dose of BPA (25 μg/L), which has an estimated intake exposure of 5 μg BPA/kg BW, increased acute CVB3 myocarditis in female BALB/c mice compared to control water. We also found that all doses of BPA increased pericarditis. We found BPA significantly decreased ERα and VDR expression, while ERβ was significantly increased in the heart at day 10 pi. At 24 hours pi, BPA increased both ERα and ERβ expression in the spleen. We also assessed protein levels and found that BPA increased phosphorylated active forms of both ERα and ERβ. We found that BPA increased mast cell (cKit) numbers and degranulation, especially along the pericardium. The main inflammatory cells increased in the heart were mast cells and CD4+ T cells according to qRT-PCR. IFN-γ, IL-17, C5aR1, TLR4, caspase-1, IL-1β, and Mmp9 were all significantly increased in the heart during myocarditis with BPA exposure, all immune components known to increase myocarditis and

remodeling. Thus, BPA exposure at a clinically relevant dose is able to increase CVB3-induced myocarditis in female BALB/c mice housed in traditional plastic cages and water bottles.

Introduction

Myocarditis is an inflammatory heart disease that leads to DCM and heart failure (Cooper 2009A). Myocarditis has been found to be influenced by the sex hormones testosterone and estrogen, which likely accounts for the increased prevalence and severity of disease in men over women (Lyden 1987, Frisancho-Kiss 2006B, Frisancho-Kiss 2007, Cocker 2009, McNamara 2011, Coronado 2012, Fairweather 2012A, Kyto 2013). It has also been found that estrogen, via ERα but not ERβ, mediates cardioprotection in females (Regitz-Zagrosek 2008). Since myocarditis is influenced by sex hormones, EDs could play a part in the development and progression of disease. Endocrine disruptors are compounds that interfere with the function of natural hormones like estrogen (Kavlock 1997**,** Crisp 1998, Vandenberg 2009). Estrogen is known to regulate the function of the cardiovascular and immune system. The human population is exposed to BPA, a known estrogen disruptor, from plastics, such as water bottles and plastic food containers (Olea 1996, Snyder 2000, Matthews 2001, Howdeshell 2003, Hunt 2003, Jenkins 2011, Belcher 2012, Teeguarden 2013, Mirmina 2014, Oldring 2014, Berger 2015, Corrales 2015, Lorber 2015, Rahman Kabir 2015, Yama 2015, Gerona 2016, Hehn 2016, Liao 2016, Ndaw 2016). BPA is an estrogen disruptor that mimics estrogen, acting as an agonist that binds to the ER (Kuiper 1997, Anderson 1999, Hiroi 1999, Molina-Molina 2013, Li 2015, Zaid 2015). BPA has been found to act through ERβ in cardiomyocytes to increase cardiac arrhythmias (Liang 2014, Belcher 2015). Thus, BPA could increase myocarditis through deleterious actions of the ERβ signaling rather than beneficial effects via ERα signaling. To our knowledge no one has examined the role of EDs like BPA on myocarditis or DCM. Previous studies by us and others have established that CVB3-induced myocarditis is increased by testosterone (Huber 1994, Gauntt 2003,

Frisancho-Kiss 2006A, Frisancho-Kiss 2006, Frisancho-Kiss 2007, Frisancho-Kiss 2009, Coronado 2012, Fairweather 2012A, Fairweather 2013, Reddy 2013), while BPA down-regulates the protective effects of estrogen (Matthews 2001). This raises the possibility that myocarditis could be influenced by BPA exposure, which is an estrogenic endocrine disruptor (Belcher 2012).

To test this idea, we exposed adult female BALB/c mice to different doses of BPA dissolved in MilliQ water or control drinking water (0 μg/L water) for 2 weeks using traditional plastic cages and water bottles (**Chapter 2, Table 3**), prior to ip injection with CVB3 to induce myocarditis and tissues were harvested at day 10 pi during peak myocarditis (Meyers 2013). BPA exposure was continued from day 0 of infection until harvest at day 10 pi. Soy-free food and bedding were used to prevent that estrogenic influence on the experiments. The BPA doses used in our studies were obtained from Jenkins et al and based on predicted daily exposure levels in the human population (Jenkins 2011). The doses that were chosen are described in **Chapter 2, Table 3**. The EPA reference dose is defined as an estimate of the daily exposure to a susceptible individual without an appreciable risk of deleterious effects during a lifetime and is equivalent to the highest dose used in our experiments (50 μg/kg/day). The EPA reference dose is calculated by using a safety factor of 1000x the LOAEL (lowest observable adverse effect level) (Vandenberg 2009).

A systematic review of recent publications on “low dose” BPA exposure in humans has a wide range of definitions for low dose. In a systematic review of 123 adult exposure studies only 13% contained a dose in the adult exposure range and of those only 3% were a human relevant oral exposure (Teeguarden 2013). Epidemiology studies define an adult external human exposure upper limit as 0.27 μg/kg/day derived from NHANES data (Lakind

2008, Lakind 2010), and for 1 month to 11 year old children an external human exposure upper limit was set at 1.61 μg/kg derived from World Health Organization data. The "low dose" exposures used for the studies in this Thesis were derived from a study by Jenkins et al which was published in 2011, before the systematic review was published in 2013 (Jenkins 2011, Teeguarden 2013). The doses we used were based on the Jenkins et al calculations of a human relevant dose of 0.5 μg/kg, a high human relevant dose of 5 μg/kg, and an EPA reference dose of 50 μg/kg (Jenkins 2011). Once we had started using these doses we continued them throughout our study for consistency.

Results

ELISA detects higher BPA levels in the drinking water of mice dosed with BPA

Levels of BPA dissolved in the drinking water were confirmed using ELISA to determine if the calculated doses correlated to detectable levels. We found a significant difference between groups (p =0.04) (**Fig 1**). There were significantly higher levels of BPA in the 25 (p=0.02) and 250 (p= 0.01) μg/L doses of water compared to control water (**Fig 1**). Although the BPA levels determined by ELISA were significantly increased at higher doses, the BPA concentrations determined by the ELISA did not match the mathematically calculated doses. This is not surprising because ELISA is considered to be a poor method for accurately determining BPA levels in water or sera. However, at this stage of the research project we had not yet developed collaborators who could assess BPA levels using liquid chromatography-mass spectrometry/ mass spectrometry (LC-MS/MS).

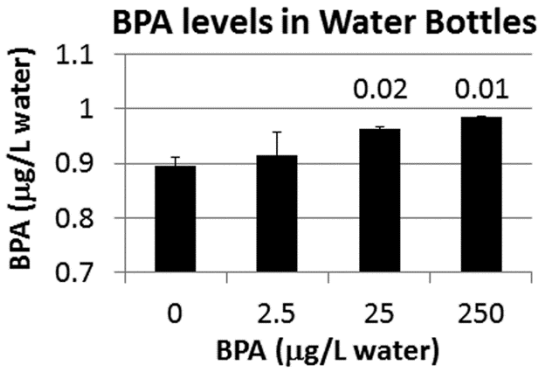


Figure 1. BPA concentration in water bottles was increased in higher doses of BPA when measured by ELISA. BPA levels were assessed in water bottles dosed with BPA prior to treating mice; x-axis represents BPA levels used for exposure and y-axis BPA levels determined by ELISA. The ELISA was performed according to the manufactures' instructions. Data show the mean +/-SEM using 3-4 samples obtained from water bottles/ group. Significance was calculated using one-way

ANOVA (p=0.04) followed by one-way Student's t tests comparing individual BPA dilutions to 0 control group.

BPA exposure increases CVB3 myocarditis in female BALB/c mice

Studies have found that BPA exposure increases cardiac, autoimmune and inflammatory diseases such as cardiac arrhythmias (Orton 2004, Weinstock-Guttman 2003, Kendziorski 2012, Melzer 2012, Shankar 2012, Gao 2014, O'Brien 2014, Jochmanova 2015, Ranciere 2015, Han 2016, He 2016). Sex hormones like testosterone are known to influence cardiac diseases such as CVB3 myocarditis (Frisancho-Kiss 2009, Coronado 2012). To assess the effect of BPA exposure on CVB3 myocarditis, 6-8 week old female BALB/c mice were given varying doses of BPA in their drinking water for two weeks prior to infecting mice with CVB3 ip on day 0. Mice were housed in typical plastic cages using plastic water bottles, but were provided bedding and food free of soy which contains genistein, an estrogenic agent. The doses of BPA that were administered were 2.5, 25, and 250 μg BPA/L in drinking water, which is equivalent to an estimated intake of 0.5, 5, and 50 μg BPA/kg body weight (BW), respectively, based on daily water intake by Jenkins et al. Examining the dose response of BPA was important to determine whether there was a dose effect on myocarditis. BPA treatment was continued from day 0 after infection until the harvest at day 10 pi. Myocarditis was assessed histologically at day 10 pi, during acute myocarditis, by determining % inflammation in heart sections corrected for the size of the heart.

We found that BPA exposure of mice increased CVB3-induced myocarditis (p=0.01). Controlling for multiple comparisons revealed that the 25 μg BPA/L dose, which is equivalent to an estimated intake of 5 μg BPA/kg BW, significantly increased myocarditis at

day 10 pi compared to control water containing no supplemental BPA (p<0.01) (**Fig 2**). Representative photos of the effect of BPA exposure on CVB3 myocarditis are shown in **Figure 3**. Although the 50 μg/kg dose of BPA was found to be significant compared to the 0 BPA control (p=0.04), this dose was no longer significant when multiple comparisons were taken into consideration. These data suggest that exposure to BPA is not demonstrating a dose-effect, but that the estimated "high human exposure" dose (25 μg BPA/L water or 5 μg BPA/kg BW) increases myocarditis. Future studies will assess the effect of BPA on inflammation without viral infection to determine if co-exposure is nessessary.

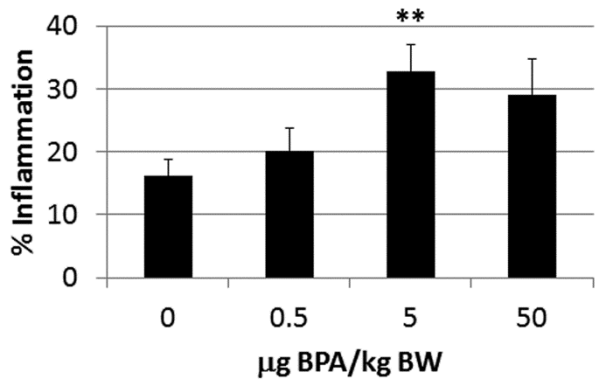


Figure 2. BPA increases myocarditis in BALB/c female mice in plastic cages. Female BALB/c mice were given increasing doses of bisphenol A (BPA) in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and inflammation examined at day 10 pi, during peak myocarditis. BPA exposure continued from day 0 to 10 pi. Myocarditis was assessed as the % inflammation in the heart using H&E compared to the overall size of the heart section by histology using an eyepiece grid. Estimated BPA intakes were determined as follows: 0, 0.5, 5, and 50 μg BPA/kg body weight (BW) were obtained by dosing water with 0, 2.5, 25 or 250 μg BPA/L, respectively. Data show the mean +/-SEM, n=9-20/group. One-way ANOVA found a significant difference existed between groups (p=0.01). After controlling for multiple comparisons, the 5 μg BPA/kg BW dose was significantly increased compared to control water (**, p<0.01).

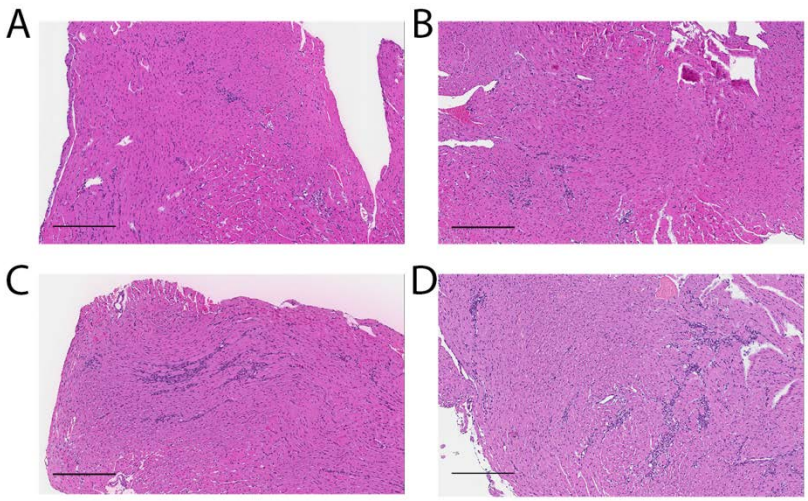


Figure 3. Representative photos of the effect of BPA exposure on CVB3 myocarditis. Female BALB/c mice were given increasing doses of bisphenol A (BPA) in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and inflammation examined at day 10 pi, during peak myocarditis. BPA exposure continued from day 0 to 10 pi. Inflammation in the heart was assessed using H&E stain where cardiac tissue appears pink and inflammation dark purple. Representative photos depict **A)** 0, **B)** 0.5, **C)** 5, and **D)** 50 μg BPA/kg BW. The bar is 300 μm in length.

BPA exposure increases pericarditis in female BALB/c mice after CVB3 infection

Myocarditis in patients often includes inflammation and/or damage of the pericardium, a single layer of cells surrounding the heart, which is called pericarditis (Imazio 2013). When myocarditis and pericarditis occur together it is often termed myopericarditis or perimyocarditis (used interchangeably) (Imazio 2013). Male mice in our CVB3 myocarditis model also often develop pericarditis/ myopericarditis (Fairweather 2004A, Fairweather 2006). Representative photos of pericarditis are shown in **Figure 4**. Normal pericardium consists of one layer of pericardial cells on the outer surface of the myocardium as depicted

in **Figure 4A**. Damaged pericardium lifts from the myocardium with a "lacey-like" appearance (**Fig 4B**). More severe pericarditis develops multiple layers with or without inflammatory cells (**Fig 4C-D**). In this study we found that BPA exposure increased the level of pericarditis and/or pericardial damage during CVB3 myocarditis (one-way ANOVA, p=0.002) (**Fig 5**). Controlling for multiple comparisons revealed that the 5 and 50 µg BPA/kg BW doses significantly increased pericarditis at day 10 pi compared to control water (p<0.05 and p<0.001, respectively) (**Fig 5**). These data indicate that BPA exposure at the estimated "high human exposure" and "EPA reference" dose increase CVB3-induced pericarditis.

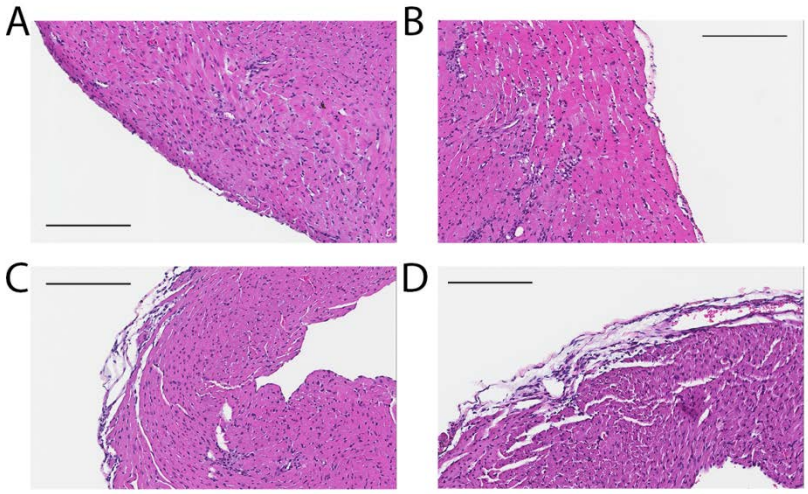


Figure 4. Representative photos of the effect of BPA on CVB3 pericarditis in female mice. Female BALB/c mice were given increasing doses of bisphenol A (BPA) in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and pericarditis examined at day 10 pi. BPA exposure continued from day 0 to 10 pi. Pericarditis was assessed using H&E stain. Representative photos depict **A)** 0, **B)** 0.5, **C)** 5, and **D)** 50 µg BPA/kg BW. The bar is 200 µm in length. Normal pericardium consists of one layer of cells on the outer surface of the myocardium as seen in **A)** above.

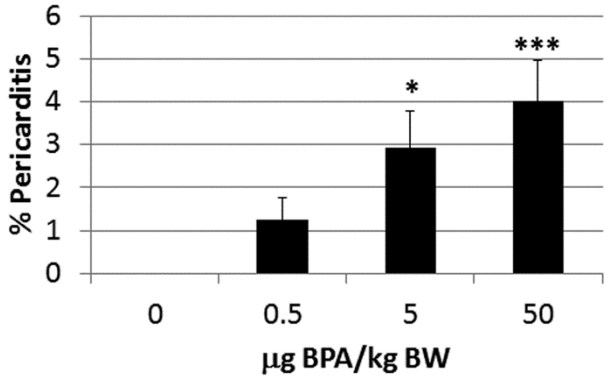


Figure 5. BPA increases pericarditis in BALB/c female mice. Female BALB/c mice were given increasing doses of bisphenol A (BPA) in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and inflammation examined at day 10 pi, during peak myocarditis. BPA exposure continued from day 0 to 10 pi. Pericarditis was assessed as follows: pericarditis that ran through 3 grid squares in the eyepiece grid was counted as a value of 1. The % pericarditis was calculated as the number of sets of 3 squares with pericarditis (values ranging from 1 to 6 in a typical heart section) compared to the overall size of the heart section using an eyepiece grid. Estimated BPA intakes were determined as follows: 0, 0.5, 5, and 50 µg BPA/kg BW obtained by dosing water with 0, 2.5, 25 or 250 µg BPA/L, respectively. Data show the mean +/-SEM, n=9-10/group. One-way ANOVA found a significant difference existed between groups (p=0.002). After controlling for multiple comparisons, the 5 and 50 µg BPA/kg BW doses were significantly increased compared to control water (*, p<0.05; ***, p<0.001).

BPA does not significantly alter viral gene expression in the heart during myocarditis

We examined whether BPA exposure altered CVB3 replication in the heart during acute myocarditis. We used a primer/probe set directed to the CVB3 genome according to Antoniak et al (Antoniak 2013). We found that BPA had no significant effect on viral replication in the heart during acute myocarditis at day 10 pi by qRT-PCR (p=0.62) (**Fig 6**).

Thus, increased myocardial inflammation from BPA exposure was not due to increased viral replication during acute myocarditis.

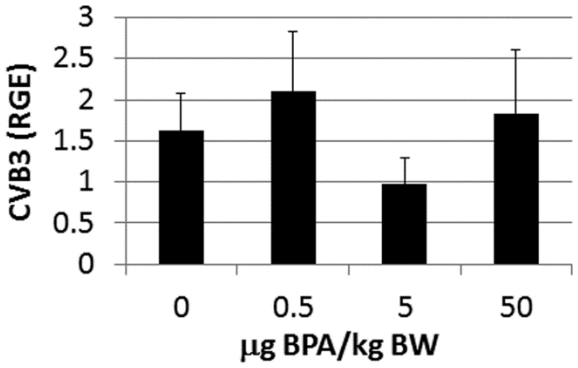


Figure 6. Viral gene expression in the heart was not effected by BPA treatment. Female BALB/c mice were given increasing doses of bisphenol A (BPA) in drinking water for 2 weeks and then injected ip with 10^3 plaque forming units (PFU) of CVB3 ip on day 0 and hearts were harvested at day 10 pi. BPA exposure continued from day 0 to 10 pi. CVB3 levels in the heart were measured using qRT-PCR to determine relative gene expression (RGE) of viral genome compared to the housekeeping gene Hprt. Data show the mean +/-SEM, *n*=9-10/group. Significant differences between groups were assessed using one-way ANOVA for multiple comparisons. Estimated BPA intakes were determined as follows: 0, 0.5, 5, and 50 µg BPA/kg BW obtained by dosing water with 0, 2.5, 25 or 250 µg BPA/L, respectively.

BPA exposure increases CD4+ T cells during CVB3 myocarditis in female mice

Because myocarditis was significantly increased only in the high human relevant dose (i.e., 5 µg BPA/kg BW) (**Fig 2**), all future analysis compared only 0 to 5µg BPA/kg BW groups. The effect of BPA exposure on specific cardiac immune cells during acute myocarditis at day 10 pi was assessed using qRT-PCR of whole hearts (i.e., not isolated cells). We found that BPA exposure of BALB/c female mice significantly increased

expression of CD4, a marker for CD4+ T cells, in the heart during CVB3 myocarditis compared to 0 BPA controls (p=0.04) (**Fig 7**). We also examined expression of CD45 (total immune cells), GR1 (neutrophils), CD11b (includes macrophages, neutrophils, mast cells and some dendritic cells), F4/80 (macrophages), CD3 (all T cells), CD8 (CD8+ T cell), and Foxp3 (regulatory T cells), but found no significant difference between 5 μg BPA and 0 BPA control water for any of these immune cell markers (**Fig 7**). Am increase in CD4+ T cells during acute myocarditis following BPA exposure is consistent with the increased inflammation observed with histology for the 5μg BPA/kg BW dose (**Fig 2**).

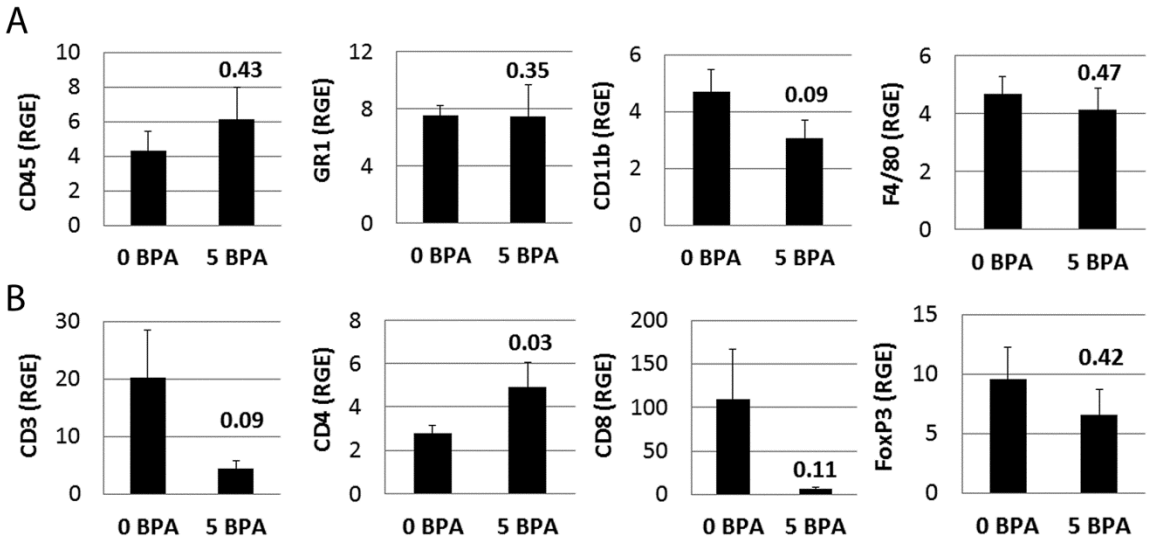


Figure 7. CD4+ T cells were significantly increased by BPA exposure. Female BALB/c mice were given 0 or 5 μg bisphenol A (0 BPA vs. 5 BPA) /kg BW in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. BPA exposure continued from day 0 to 10 pi. Immune cell populations from the whole heart were examined using qRT-PCR for relative gene expression (RGE) of the gene of interest vs. the housekeeping gene Hprt for CD45 (total immune cells), GR1 (neutrophils), CD11b (includes macrophages, neutrophils, mast cells and some dendritic cells), F4/80 (macrophages), CD3 (all T cells), CD4 (CD4+ T cells), CD8 (CD8+ T cell), and Foxp3 (regulatory T cells). Data are shown as the mean +/-SEM using a two-

tailed Student's *t* or Mann-Whitney rank test with 8-10 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA significantly increases IFNγ and IL-17 levels, an indicator of a Th1 and Th17-type immune response

BPA exposure has been shown to increase both Th1 and Th2 immune responses in various inflammatory animal models, such as the OVA model of asthma (Yoshino 2003, Yoshino 2004, Rogers 2013). Because CD4+ T cells were increased during CVB3 myocarditis by BPA exposure, we examined whether IFNγ, IL-4, and/or IL-17A (IL-17) cytokine levels were altered in the heart during acute CVB3 myocarditis following BPA exposure. Changes in these cytokines are often used to indicate whether a Th1, Th2 or Th17 response is dominant, respectively (Fairweather 2004C, Zhu 2010). We found that IFNγ was significantly increased by 5 μg/kg BW BPA exposure during acute myocarditis (**Fig 8A**), IL-4 levels were not altered (**Fig 8B**), and IL-17 was also significantly increased (**Fig 8C**). These results suggest that a Th2-type immune response was not altered by BPA exposure during CVB3 myocarditis. However, Th1 and Th17-type immune responses were significantly increased by BPA exposure during acute CVB3 myocarditis. Th1 and Th17 responses have been demonstrated to increase acute viral and autoimmune myocarditis in mice (Frisancho-Kiss 2006B, Baldeviano 2010, Wu 2014). IL-17, but not IFNγ, has been shown to drive progression from acute myocarditis to chronic myocarditis and DCM (Fairweather 2004A, Baldeviano 2010, Wu 2014).

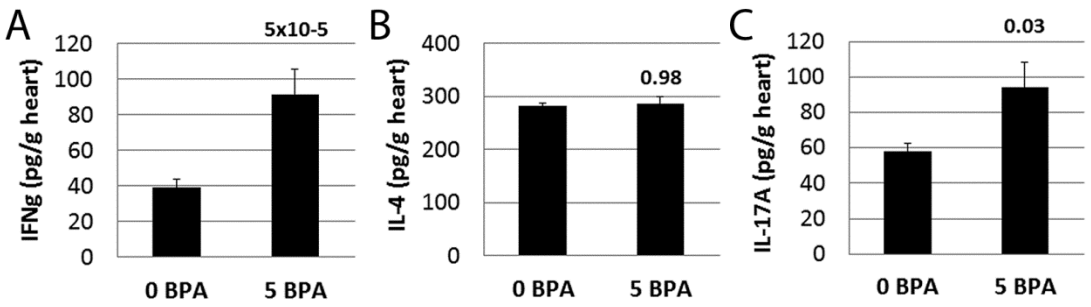


Figure 8. Cardiac IFNγ and IL-17A levels significantly increased by BPA exposure. Female BALB/c mice were given 0 or 5 μg/kg BW bisphenol A (0 BPA vs. 5 BPA) in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0. BPA exposure continued from day 0 to 10 pi. Hearts were harvested at day 10 pi and homogenized and the supernatant used to measure **A)** IFNγ, **B)** IL-4, and **C)** IL-17A levels in the heart by ELISA. Data show the mean +/-SEM using a one-tailed (IL-17A) or two-tailed Student's *t* (IFNγ and IL-4) or Mann-Whitney rank test with 10 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA does not skew the M1 vs. M2 macrophage profile in the heart during myocarditis

Classically activated M1 macrophages are driven by IFNs/Th1 responses and are increased in male BALB/c during CVB3 myocarditis, while alternatively activated M2 macrophages are anti-inflammatory and are elevated in female BALB/c mice who typically have far lower levels of myocarditis compared to males (Li 2009, Fairweather 2009, Abston 2013). Markers of M2 macrophages in mice include arginase-1 (Arg1), Ym1 (eosinophilic protein from chitinase family), and TGFβ, while M1 markers include Cxcl9 and Cxcl10 (Siracusa 2008, Fairweather 2009, Abston 2012A). Here we found that female BALB/c mice exposed to 5 μg/kg BW BPA had no significant change in the anti-inflammatory M2 macrophage markers arginase-1, Ym1, TGFβ1 (0 BPA 1.29 +/-0.06 vs. 5 PBA 1.43 +/-0.1, p=0.61) or TGFβ2 (0 BPA 2.89+/-0.38 vs. 5 BPA 3.33+/-0.07, p=0.74) (**Fig 9A**) or the M1

markers Cxcl9 or Cxcl10 (**Fig 9B**) compared to 0 BPA control water by qRT-PCR. These data suggest that the elevated IFNγ in the heart from BPA exposure is derived from T cells rather than macrophages (Darwich 2009), and that the elevated IFNγ in response to BPA is not significantly shifting the M1/M2 profile of cardiac macrophages during CVB3 myocarditis.

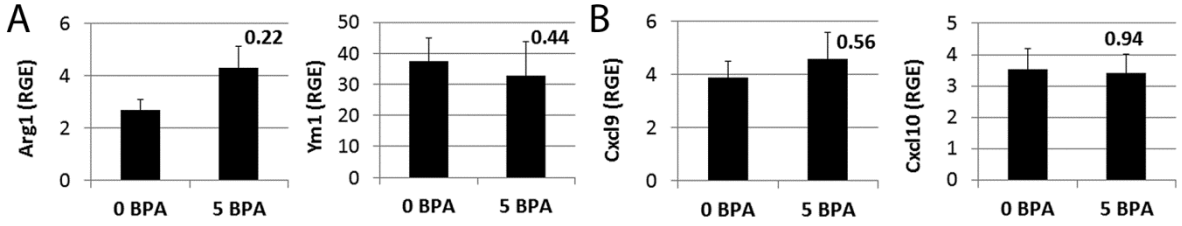


Figure 9. BPA exposure does not alter M1 vs. M2 macrophage profile during myocarditis. Female BALB/c mice were given 0 or 5 μg/kg BW BPA (0 BPA vs. 5 BPA) in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0. BPA exposure continued from day 0 to 10 pi. Hearts were harvested at day 10 pi during acute myocarditis. Relative gene expression (RGE) of **A)** M2 macrophage markers (Arg1, Ym1) and **B)** M1 markers (Cxcl9, Cxcl10) vs. Hprt controls were assessed in whole hearts using qRT-PCR at day 10 pi comparing 0 and 5 μg BPA/kg BW groups. Data show the mean ±SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 9-10 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA exposure increases the number of mast cells in the heart during myocarditis

Previously, BPA exposure was found to increase mast cell activation and numbers in *in vitro* studies (O'Brien 2014A, O'Brien 2014B). The effect of BPA exposure on cardiac mast cells during acute myocarditis at day 10 pi was assessed using toluidine blue stain with histology, which detects mast cell granules, and qRT-PCR for cKit (also called stem cell factor/SCF). We found that 5 μg BPA/ kg BW exposure to BALB/c female mice in plastic

cages significantly increased the total number of mast cells in the heart by histology (p=0.007) (**Fig 10A**), and significantly increased cKit (CD117) expression in whole hearts by qRT-PCR (p=0.004) (**Fig 10B**) compared to 0 BPA control water during CVB3 myocarditis. cKit is also called SCF and a marker used to detect both mast cells and stem cells (Draber 2016, Huang 2016). Thus, exposure of BPA to female BALB/c mice may increase myocarditis by increasing the number of mast cells in the heart during acute myocarditis.

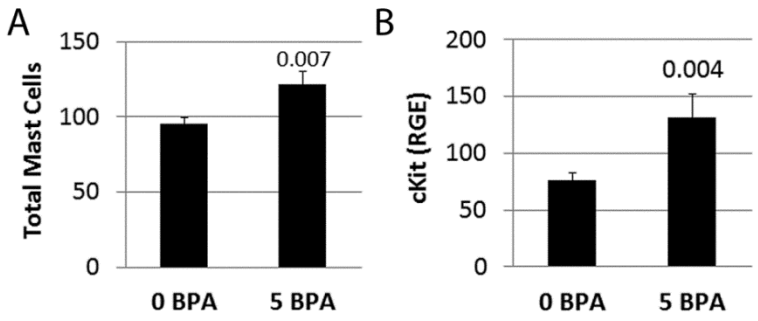


Figure 10. BPA exposure increases the number of cardiac mast cells. Female BALB/c mice were given 0 or 5 μg/kg BW bisphenol A (0 BPA vs. 5 BPA) in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0. BPA exposure continued from day 0 to 10 pi. Hearts were harvested at day 10 pi during acute myocarditis. **A)** Toluidine blue was used to detect mast cells (detects mast cell granules) by histology and the number of mast cells were normalized to the size of the heart using an eyepiece grid. **B)** Relative gene expression (RGE) of CD117 (cKit) was used to detect mast cell numbers using qRT-PCR of whole hearts vs. HPRT controls at day 10 pi comparing 0 and 5 μg BPA/kg BW groups. Data show the mean ±SEM using a two-tailed Student's t or Mann-Whitney rank test with 10-20 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA exposure increases degranulating mast cells in the heart during myocarditis

Previously, we found that mast cell degranulation was associated with increased CVB3 myocarditis, pericarditis, DCM and heart failure in mice (Fairweather 2004A, Fairweather 2006). In order to determine whether BPA exposure affected mast cell degranulation during CVB3 myocarditis in BALB/c female mice we counted the number of degranulating vs. non-degranulating mast cells using toluidine blue-stained histology sections. **Figure 11** shows representative examples of non-degranulating vs. degranulating mast cells stained with toluidine blue.

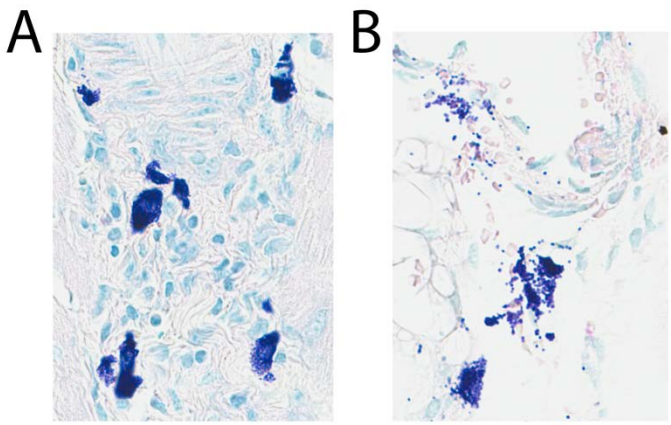


Figure 11. Example of non-degranulating and degranulating mast cells. Toluidine blue was used to detect cardiac mast cells with histology by staining mast cell granules purple. Cell nuclei appear pale blue. Eosin (pink) was used as a counterstain to detect cardiac tissue. Red blood cells also appear pink. A representative example of a **A)** non-degranulating (dense granules within the cell) and **B)** degranulating (cells have the appearance as if they had "exploded") mast cells are shown. Magnification approximately 400x.

We found that during acute myocarditis more mast cells were not degranulating (i.e., intact) compared to those that were degranulating ($p<0.0001$) (**Fig 12**), which is typical in our experience with this model. BPA was found to have a significant effect on increasing

mast cells in the heart overall (degranulating and non-degranulating) (p=0.0006) (**Fig 12**), confirming the data shown in **Figure 10A**. An exposure of 5 μg BPA/kg BW (5 BPA) had no significant effect on mast cells that were not degranulating (NOT), but significantly increased the number of degranulating mast cells (Degran) during myocarditis (p<0.05) compared to the 0 BPA group (**Fig 12**).

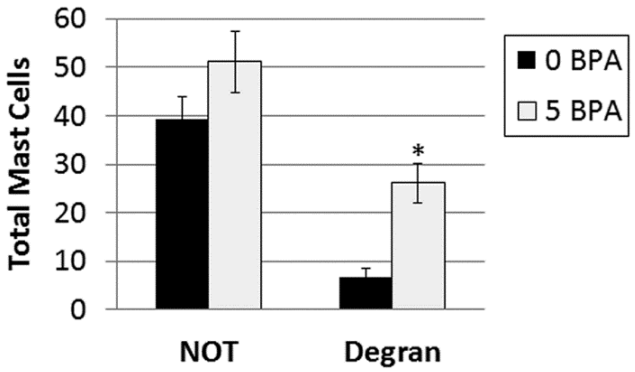


Figure 12. BPA exposure increases the number of degranulating cardiac mast cells. Female BALB/c mice were given 0 or 5 μg/kg BW bisphenol A (0 BPA vs. 5 BPA) in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0. BPA exposure continued from day 0 to 10 pi. Hearts were harvested at day 10 pi during acute myocarditis. Toluidine blue was used to detect mast cells (detects mast cell granules) by histology and the number of mast cells that were not degranulating (NOT) (i.e., intact) or degranulating (Degran) for each group were normalized to the size of the heart using an eyepiece grid. Data show the mean ±SEM with 10 mice/ group using 2-way ANOVA with Tukey's multiple comparisons test. Two-way ANOVA found significant results for degranulation p<0.0001 and BPA p=0.0006. The 5 BPA exposure significantly increased the number of degranulating mast cells during myocarditis compared to the 0 BPA group (*, p<0.05). 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA exposure increases pericardial mast cell degranulation during myocarditis

Previously, we found that *pericardial* mast cell degranulation was associated with increased pericarditis, immune complex deposition along the pericardium, DCM and heart failure in mice (Fairweather 2004A, Fairweather 2006). In order to determine whether BPA exposure affected *pericardial* mast cell degranulation during CVB3 myocarditis in BALB/c female mice we counted the number of degranulating (Degran) vs. non-degranulating (NOT) mast cells that were located on the outer pericardial layer of the heart using toluidine blue-stained histology sections. We found that during acute myocarditis more *pericardial* mast cells were not degranulating (i.e., intact) compared to those that were degranulating ($p<0.0002$) (**Fig 13, Fig 14**). BPA was found to have a significant effect on increasing *pericardial* mast cells in the heart overall (degranulating and non-degranulating) ($p=0.0004$) (**Fig 13**). An exposure of 5 μg BPA/kg BW (5 BPA) had no significant effect on *pericardial* mast cells that were not degranulating, but significantly increased the number of degranulating *pericardial* mast cells during myocarditis ($p<0.01$) compared to the 0 BPA group (**Fig 13**). In contrast to total degranulating mast cells (**Fig 12**), *pericardial* mast cell degranulation was less only without BPA exposure (0 BPA group) compared to non-degranulating *pericardial* mast cells ($p<0.0001$) (**Fig 13**). This indicates that BPA exposure strongly increases *pericardial* mast cell numbers, regardless of degranulation state.

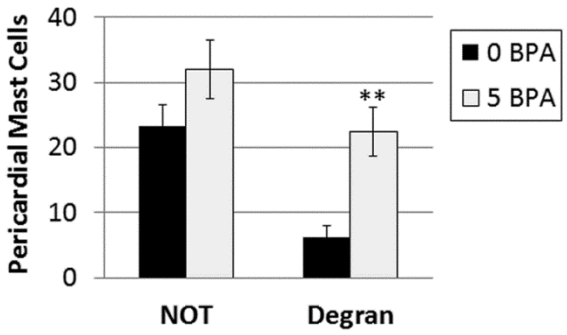


Figure 13. BPA exposure increases the number of degranulating *pericardial* mast cells. Female BALB/c mice were given 0 or 5 μg/kg BW bisphenol A (0 BPA vs. 5 BPA) in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0. BPA exposure continued from day 0 to 10 pi. Hearts were harvested at day 10 pi during acute myocarditis. Toluidine blue was used to detect mast cells (detects mast cell granules) by histology and the number of *pericardial* mast cells that were not degranulating (NOT) (i.e., intact) or degranulating (Degran) for each group were normalized to the size of the heart using an eyepiece grid. Data show the mean ±SEM with 10 mice/ group using 2-way ANOVA with Tukey's multiple comparisons test. Two-way ANOVA found significant results for degranulation $p<0.0002$ and BPA $p=0.0004$. BPA significantly increased the number of *pericardial* degranulating mast cells during myocarditis compared to the 0 BPA group (**, $p<0.01$). 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

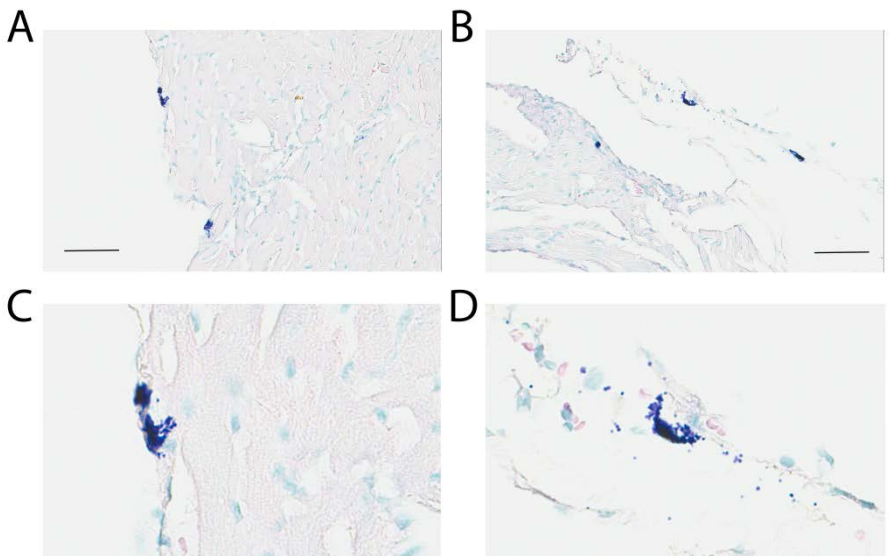


Figure 14. Example of non-degranulating and degranulating *pericardial* mast cells. Toluidine blue was used to detect *pericardial* mast cells with histology by staining mast cell granules purple. Cell nuclei appear pale blue. Eosin (pink) was used as a counterstain to detect cardiac tissue. Red blood cells also appear pink. A representative example of a **A&C)** non-degranulating *pericardial* and **B&D)** degranulating *pericardial* mast cells are shown. **A&B)** Bar is 60mm in length. **C&D)** Magnification 400x.

BPA exposure does not alter degranulation of myocardial mast cells during myocarditis

Mast cells leave the bone marrow as undifferentiated cells and migrate to the heart where they differentiate to form two types of resident mast cell populations: one type contains tryptase and chymotrypsin (also called Serpin A3n) granules and is termed a *TC mast cell* and the other type of mast cell contains only tryptase granules and is termed a *T mast cell* (Irani 1986, Fairweather 2008B). It is not known whether one type of mast cell resides along the pericardium and the other in the myocardium, but this is a possibility. To determine whether BPA exposure altered degranulation of mast cells located in the myocardium we examined the degranulation state of myocardial mast cells using histology. A representative photo of pericardial vs. myocardial mast cells is shown in **Figure 15**.

By 2-way ANOVA we found that during acute myocarditis more *myocardial* mast cells were not degranulating (i.e., intact) compared to those that were degranulating ($p<0.0001$) (**Fig 16**). BPA did not increase *myocardial* mast cells in the heart overall ($p=0.71$) (**Fig 16**). Two-way ANOVA and a Student's *t* test ($p=0.09$) comparison of 0 BPA to 5 BPA degranulating *myocardial* mast cells did not find a significant difference in degranulation. These data further indicate that BPA exposure primarily affects *pericardial* mast cells.

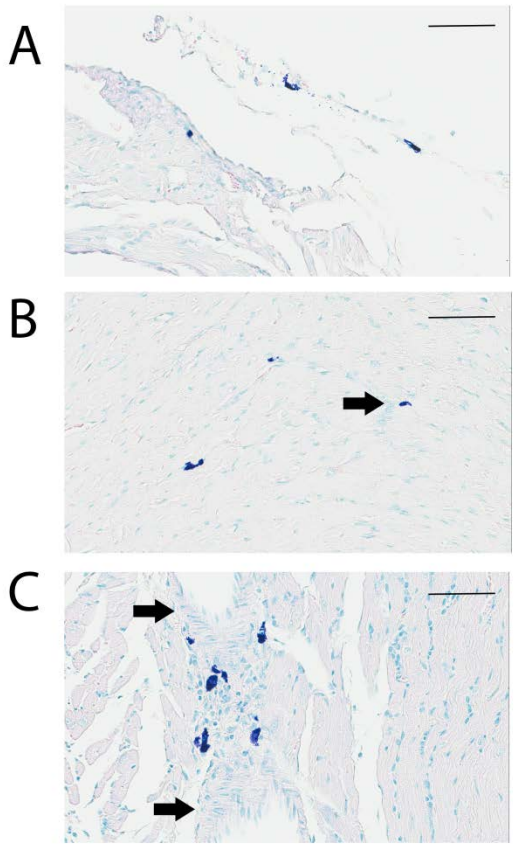


Figure 15. Example of pericardial, myocardial and vessel-associated mast cells. Toluidine blue was used to detect cardiac mast cells by location with histology by staining their granules purple compared to the eosin counterstain of tissue cells, which is pink. Nuclei of all types of cardiac cells stain a pale blue. Representative examples of **A)** pericardial, **B)** myocardial, and **C)** vessel-associated mast cells are shown. **B)** If mast cell is not near a vessel or red blood cells it was counted as a "myocardial" mast cell (*arrow*). **C)** Arrows indicate vessels located above and below purple stained mast cells. Bar is 60mm in length.

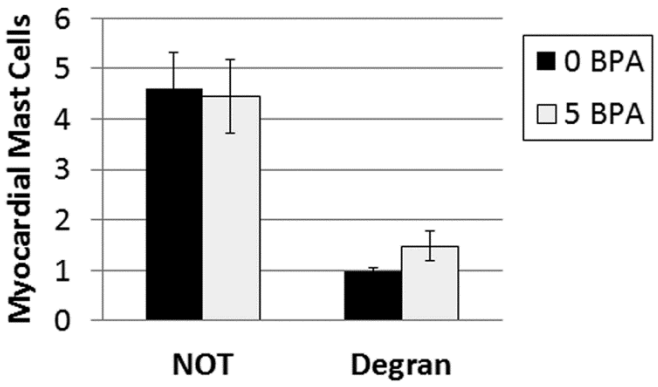


Figure 16. BPA exposure does not alter *myocardial* mast cell numbers during myocarditis. Female BALB/c mice were given 0 or 5 μg/kg BW bisphenol A (0 BPA vs. 5 BPA) in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0. BPA exposure continued from day 0 to 10 pi. Hearts were harvested at day 10 pi during acute myocarditis. Toluidine blue was used to detect mast cells (detects mast cell granules) by histology and the number of *myocardial* mast cells that were not degranulating (NOT) or degranulating (Degran) for each group were normalized to the size of the heart using an eyepiece grid. Data show the mean ±SEM with 10 mice/ group using 2-way ANOVA with Tukey's multiple comparisons test. Two-way ANOVA found significant results for degranulation only *(*p<0.0001). 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA exposure may increase degranulation of "vessel-associated" mast cells

Mast cells are released from bone marrow and reside in tissues. Basophils remain in the circulation. Basophils can be recruited to sites of inflammation (Otsuka 2016). Infiltrating mast cells or basophils would be expected to be found near vessels. Mast cells found near vessels have been termed "vessel-associated" mast cells for this analysis and are assumed to be mast cells recruited to the heart from the circulation rather than resident mast cells. Mast cells in the myocardium that were not near vessels or evidence of red blood cells were

defined as "myocardial" mast cells. It is likely that there is a certain amount of overlap between "myocardial" and "vessel-associated" mast cells due to the inability to detect vessels depending on the cut of the histology section. Representative photos of pericardial, myocardial, and vessel-associated mast cells are shown in **Figure 15**. To determine whether BPA exposure altered degranulation of mast cells located near vessels we examined the degranulation state of "vessel-associated" mast cells using histology.

We found that during acute myocarditis more "vessel-associated" mast cells were not degranulating compared to those that were degranulating regardless of BPA exposure (p<0.0001) (**Fig 17**). BPA did not increase "vessel-associated" mast cells in the heart overall (degranulating and non-degranulating) (p=0.11) (**Fig 17**). By 2-way ANOVA there was no significant difference between the 0 BPA and 5 BPA groups for "vessel-associated" degranulating mast cells (**Fig 17**). However, a Student's t test comparison of 0 BPA to 5 BPA degranulating "vessel" mast cells suggests that BPA may increase degranulation of these cells (p=0.005). These data raise the possibility that BPA exposure may increase degranulation of mast cells near vessels during myocarditis.

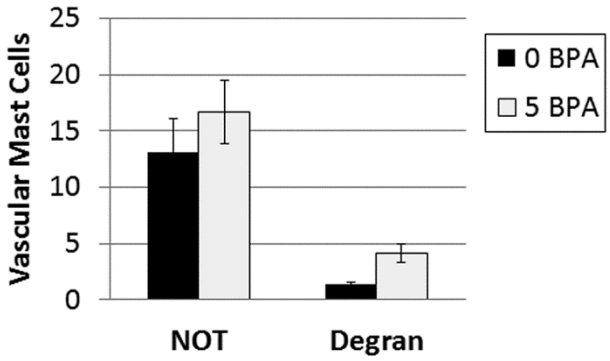


Figure 17. BPA exposure does not significantly alter "vessel-associated" mast cell numbers during myocarditis. Female BALB/c mice were given 0 or 5 μg/kg BW bisphenol A (0 BPA vs. 5 BPA) in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0.

BPA exposure continued from day 0 to 10 pi. Hearts were harvested at day 10 pi during acute myocarditis. Toluidine blue was used to detect mast cells (detects mast cell granules) by histology and the number of "vessel-associated" or "vascular" mast cells that were not degranulating (NOT) or degranulating (Degran) for each group were normalized to the size of the heart using an eyepiece grid. Data show the mean ±SEM with 10 mice/ group using 2-way ANOVA with Tukey's multiple comparisons test. Two-way ANOVA found significant results for degranulation only *(p*<0.0001). However, a Student's *t* test comparison of 0 BPA to 5 BPA degranulating "vessel/vascular" mast cells suggests that BPA may increase degranulation of these cells (*p*=0.005). 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA exposure increases expression of the mast cell anaphylatoxin receptor C3aR1

Coxsackievirus infection activates the complement pathway, which in turn activates mast cells and macrophages via CD11b, also known as complement receptor 3 (CR3), leading to increased inflammation (Fairweather 2006, Fairweather 2008B, Onyimba 2011, Coronado 2012). In addition to the complement receptor CD11b, mast cells are also activated by the mast cell-specific anaphylatoxin receptors C3aR and C5aR (Arbore 2016). Although complement activation of mast cells would be expected to peak during the innate immune response (hours and first few days after infection), we have found complement activation in the heart during CVB3 myocarditis (Fairweather 2006). Because we had found that BPA exposure increased mast cell numbers (**Fig 10**) and degranulation (**Fig 12, Fig 13**), we examined whether the complement anaphylatoxin receptors, which are specific for mast cells, were altered by BPA. We found that BPA exposure significantly increased expression of the mast cell anaphylatoxin receptor C3aR1 during acute myocarditis by qRT-PCR of whole hearts (*p*=0.04) (**Fig 18A**), but not C5aR1 (**Fig 18B**) or the complement receptor

ligands C3 (**Fig 18C**) and C4b (**Fig 18D**) at day 10 pi. Elevation of C3aR1 expression in the heart during acute myocarditis following BPA exposure is a further indication that BPA activates mast cells.

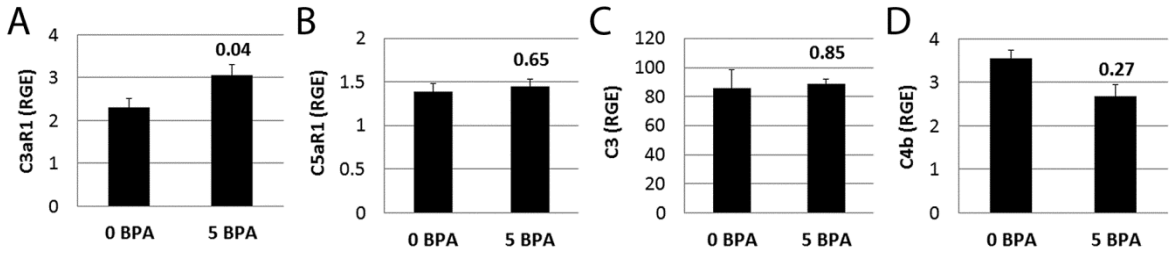


Figure 18. BPA exposure activates the mast cell anaphylatoxin receptor C3aR1. Female BALB/c mice were given 0 or 5 μg/kg BW bisphenol A (0 BPA vs. 5 BPA) in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0. BPA exposure continued from day 0 to 10 pi. Hearts were harvested at day 10 pi during acute myocarditis. Relative gene expression (RGE) of complement genes vs. the housekeeping control Hprt was examined for **A)** C3aR1, **B)** C5aR1, **C)** C3 and **D)** C4b mRNA by qRT-PCR at day 10 pi comparing 0 to 5 μg BPA/kg BW estimated intake groups. Data show the mean +/-SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 7-10 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA exposure decreases CR1, an inhibitor of complement activation on mast cells

CR1 is the primary receptor in the heart that regulates complement activation on macrophages and mast cells (Fairweather 2006, Alcorlo 2015). We showed previously that CR1 protects against autoimmune and viral myocarditis in mice (Kaya 2001, Fairweather 2006). CR1 deficient mice develop severe myocarditis, pericarditis, pericardial immune complex deposition and fibrosis and rapidly progress to DCM and heart failure following CVB3 infection, indicating the critical role for CR1 in preventing myopericarditis (Fairweather 2006). For this reason we examined CR1 expression in the heart during

myocarditis following BPA exposure using qRT-PCR. In humans, separate genes code for CR1 and CR2 on immune cells (Kurtz 1990). In mice, CR1 and CR2 are the result of alternative mRNA splicing of the same gene so that CR1 and CR2 gene expression cannot be readily distinguished in mice using qRT-PCR. CR1 regulates/inhibits complement activation on mast cells and macrophages, whereas CR2 is primarily expressed on B cells and activates B cells in response to complement (Fairweather 2006, Roosendaal and Carroll 2007, Jacobson and Weis 2008).

In this study, we found that CR1/2 expression in the heart was significantly decreased by exposure to BPA during acute CVB3 myocarditis (p=0.02) (**Fig 19A**). CR2 forms a B cell receptor complex with CD19 and CD81 is needed for complement activation of B cells. So we examined the level of CD19 by qRT-PCR, a marker often used for gene expression to detect B cells. We found that CD19 was also significantly decreased by BPA exposure during acute myocarditis (p=0.03) (**Fig 19B**), which indicates that BPA decreases CR2 leading to reduced numbers and/or activation of B cells. These data show that BPA downregulates CR1, which could contribute to increased mast cell activation (i.e., degranulation) with viral and BPA co-exposure.

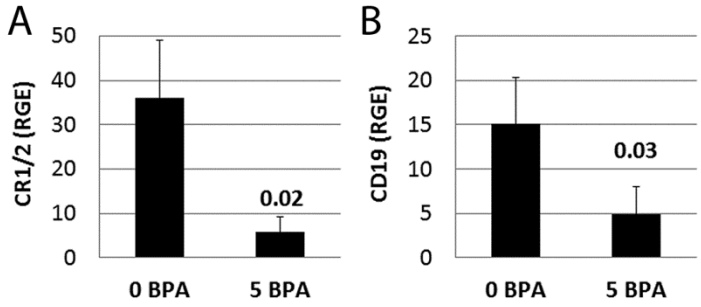


Figure 19. BPA exposure decreases CR1 and CD19/CR2 expression in the heart during myocarditis. Female BALB/c mice were given 0 or 5 μg/kg BW bisphenol A (0 BPA vs. 5 BPA) in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0. BPA

exposure continued from day 0 to 10 pi. Hearts were harvested at day 10 pi during acute myocarditis. Relative gene expression (RGE) of **A)** CR1/2 and **B)** CD19 (part of the complement-activated CR2 signaling complex on B cells) were compared to Hprt controls in the heart by qRT-PCR at day 10 pi comparing 0 to 5 μg BPA/kg BW estimated intake groups. Data show the mean +/-SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 7-9 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA treatment increases TLR4/IL-1R/caspase-1/IL-1β pathway activation in the heart during myocarditis

We showed previously that components of IL-1R-mediated signaling (i.e., TLR4, IL-1R antagonist, caspase-1, IL-1β and IL-18) are upregulated in the heart during CVB3 myocarditis in mice and that testosterone elevates this pathway on mast cells and macrophages during the innate and adaptive immune response to CVB3 (Frisancho-Kiss 2006A, Frisancho-Kiss 2007, Frisancho-Kiss 2009, Coronado 2012, Roberts 2013, Fairweather 2015). In this study we found that BPA exposure significantly increased expression of components of this pathway in the heart during acute CVB3 myocarditis in female BALB/c mice (**Fig 20**). We found that TLR4 (p=0.01), caspase-1 (p=0.001) and IL-1β (p=0.009) levels were significantly increased by BPA exposure compared to 0 BPA control water (**Fig 20**). IL-1R2, which is part of the IL-1R/TLR complex, was not significantly different between groups (p=0.06) (**Fig 20**). Expression of ST2 receptor, which is part of the TLR/IL-1R signaling family and expressed on mast cells and macrophages, was also significantly increased in the heart by BPA exposure during myocarditis using qRT-PCR (0 BPA 2.99+/-0.86 vs. 5 BPA 4.55+/-0.47, p=0.04).

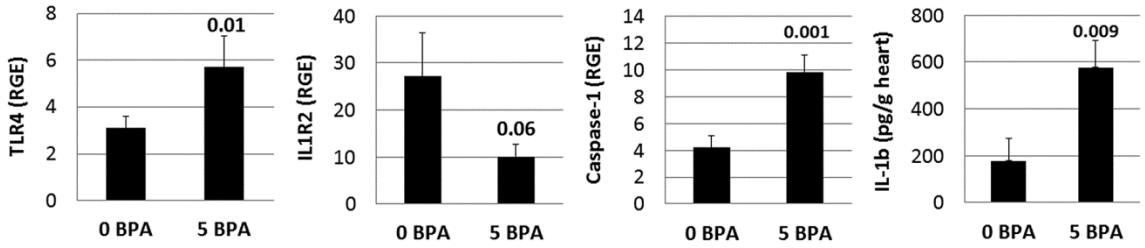


Figure 20. BPA exposure activates TLR4 pathway genes during myocarditis. Female BALB/c mice were given 0 or 5 μg/kg BW bisphenol A (0 BPA vs. 5 BPA) in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0. BPA exposure continued from day 0 to 10 pi. Hearts were harvested at day 10 pi during acute myocarditis. Relative gene expression (RGE) of genes vs. the housekeeping control Hprt for TLR4, IL-1R2, and caspase-1 were examined in whole hearts by qRT-PCR at day 10 pi comparing 0 to 5 μg BPA/kg BW groups. IL-1β levels were determined using ELISA from homogenized whole heart supernatants. Data show the mean +/-SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 7-10 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA alters cardiac Mmp9, IL-1β and IL-17: factors associated with cardiac remodeling during CVB3 myocarditis in male mice

We and others have shown previously that elevated TLR4/IL-1β/inflammasome activation, mast cell activation, IL-17, complement activation, reduced complement regulation by CR1, and decreased Mmp3 and Mmp9 during acute CVB3 myocarditis lead to remodeling and fibrosis and the progression to DCM and chronic heart failure in male BALB/c mice (Fairweather 2004A, Fairweather 2006, Baldeviano 2010, Coronado 2012, Wu 2014). Previously, it has been shown using knockout mice that tissue inhibitor of metalloproteinases (Timp) exacerbates remodeling during CVB3 myocarditis by regulating Mmp expression in the heart (Crocker 2007).

Because BPA exposure was found to increase the TLR4 signaling pathway, mast cell degranulation, IL-17A, complement activation, and reduced complement regulation by CR1, we examined whether BPA promoted factors associated with cardiac remodeling during acute myocarditis- a timepoint when profibrotic genes/proteins are elevated but histologic fibrosis (i.e., collagen deposition) is not yet present (Coronado 2012).We found that BPA exposure did not significantly alter expression of Timp1 or Mmp3 in the heart, but significantly decreased Mmp9 (p=0.04) (**Fig 21**), which could lead to increased remodeling in the heart. (**Fig 21A**). As shown previously, BPA exposure significantly increases the profibrotic cytokines IL-1β and IL-17A in the heart by ELISA, but did not alter transforming growth factor (TGF)β gene expression by qRT-PCR (**Fig 21B**). However, in the future TGF-β1 levels in the heart should be examined using ELISA because gene expression often does not relate to active protein levels.

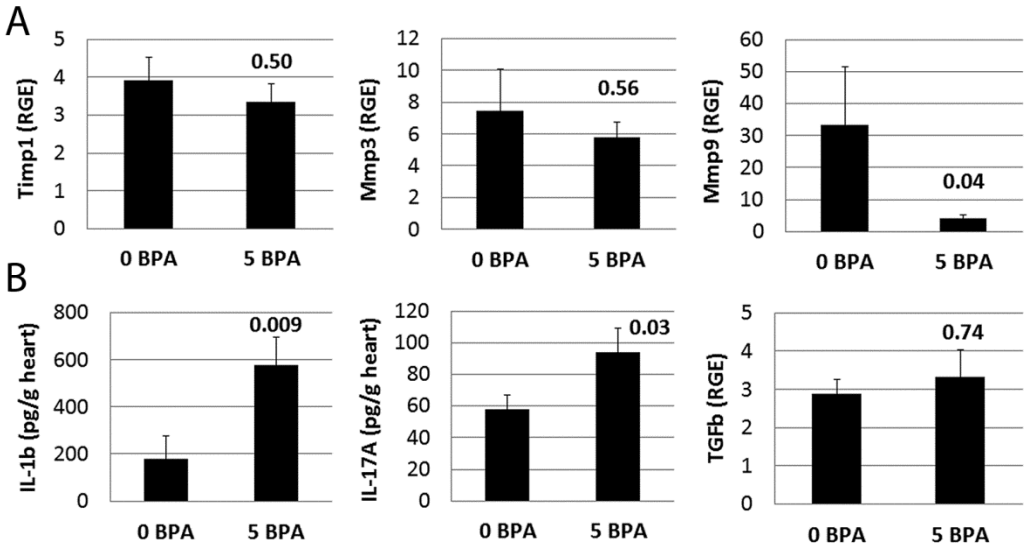


Figure 21. BPA exposure increases certain remodeling factors in the heart during myocarditis. Female BALB/c mice were given 0 or 5 μg/kg BW bisphenol A (0 BPA vs. 5 BPA) in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0. BPA exposure continued

from day 0 to 10 pi. Hearts were harvested at day 10 pi during acute myocarditis. Relative gene expression (RGE) of A) Timp1, Mmp3, Mmp9 and B) TGFβ vs. the housekeeping gene Hprt were analyzed in whole hearts by qRT-PCR at day 10 pi comparing 0 to 5 μg BPA/kg BW groups. In a separate experiment hearts were harvested at day 10 pi, homogenized and the supernatant used to measure **B)** IL-1β and IL-17A levels in the heart by ELISA. Data show the mean +/-SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 9-10 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA exposure causes increased pericardial, vascular and myocardial fibrosis during acute myocarditis

Fibrosis, assessed histologically as collagen deposition using Trichrome blue stain, is not present in the heart of male or female mice during acute CVB3 myocarditis, and only begins to appear during chronic myocarditis around day 35 pi (Fairweather 2004A, Fairweather 2005). However, remodeling genes are upregulated in the heart during acute myocarditis rather than during chronic myocarditis (Coronado 2012). Thus, typically collagen deposition, which stains bright blue with Trichrome blue staining, occurs around 2-3 weeks after upregulation of profibrotic genes in the heart. We have shown previously that this process can be accelerated and occur during acute myocarditis if certain pathways are dysregulated like CR1, TRIF or IL-33/ST2 (Fairweather 2006, Abston 2012A, Abston 2012B). Because we saw a decrease in CR1 and Mmp9 and an increase in IL-1β and IL-17A from BPA exposure- all factors that increase cardiac fibrosis, we examined whether BPA exposure could alter fibrosis in the heart during acute myocarditis. We found that that almost all mice exposed to 5 μg BPA/kg BW had increased pericardial fibrosis, shown in representative photos in **Figure 22B**. Most of the mice exposed to BPA also had greater

perivascular (**Fig 22D**) and myocardial (**Fig 23B**) fibrosis compared to mice with control water. In future studies the sections will be re-stained with Sirius red, which is easier to quantify using imaging software than Trichrome blue (Huang 2013). These data indicate that BPA exposure is able to hasten the onset of remodeling and fibrosis, which usually takes several weeks to develop. The mice had not yet developed DCM based on gross assessment of histology sections. However, pericardial fibrosis can cause changes in cardiac function that are able to be detected by echocardiography (i.e., restrictive phenotype or DCM). In future studies we will assess whether exposure to BPA led to increased cardiac dysfunction using echocardiography.

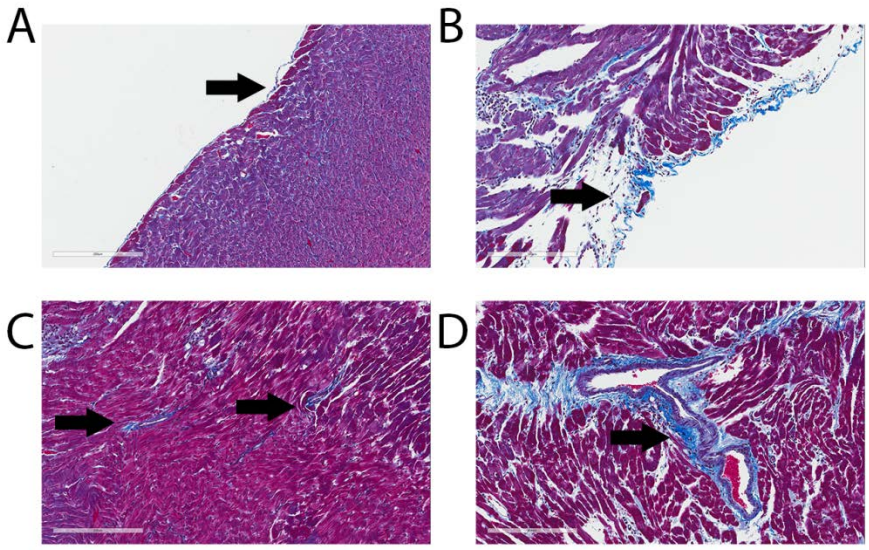


Figure 22. Representative photos of pericardial and perivascular fibrosis. Female BALB/c mice were given 0 or 5 μg/kg BW bisphenol A in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0. BPA exposure continued from day 0 to 10 pi. Hearts were harvested at day 10 pi during acute myocarditis. Fibrosis was assessed using Trichrome blue to detect collagen deposition, which stains bright blue. **A)** Normal pericardial layer; arrow points to a single layer of pericardial cells that stain blue with Trichrome. **B)** Severe pericarditis with fibrosis that stains bright blue (arrow). **C)** Normal level of perivascular fibrosis; arrows point to two vessels. **D)** Severe

perivascular fibrosis (arrow). Photos depict **A) and C)** 0 μg BPA/kg BW; **B) and D)** 5 μg BPA/kg BW. Grey bar is 200μm in length.

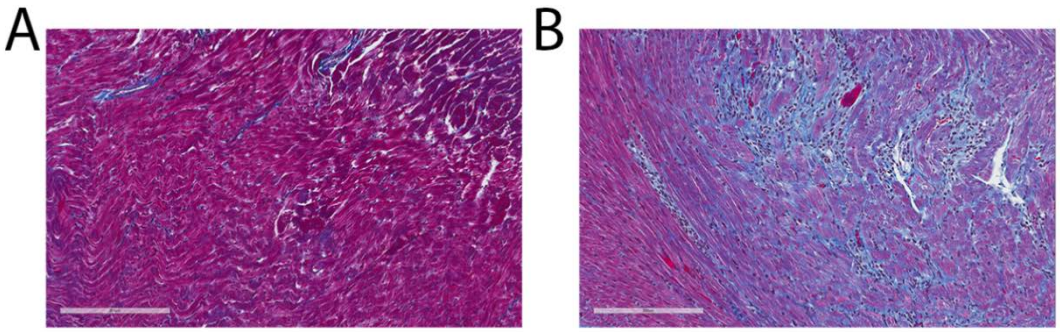


Figure 23. Representative photos of myocardial fibrosis. Female BALB/c mice were given **A)** 0 or **B)** 5 μg/kg BW bisphenol A in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0. BPA exposure continued from day 0 to 10 pi. Hearts were harvested at day 10 pi during acute myocarditis. Fibrosis was assessed using Trichrome blue to detect collagen deposition, which stains bright blue. Grey bar is 200μm in length.

BPA exposure decreases ERα and increases ERβ in the heart during CVB3 myocarditis

Estrogen receptors (ERs) are located on/in immune cells, cardiomyocytes, endothelial cells, and cardiac fibroblasts (Regitz-Zagrosek 2008, Vitale 2009, Gilliver 2010, Villablanca 2010, Buskiewicz 2016). ERα is believed to mediate most of the cardioprotective effects of estrogen in women based on studies in patients and animal models of hypertrophy, DCM and fibrosis (Regitz-Zagrosek 2008). In contrast, ERβ has been shown to have negative effects on cardiac function during these diseases in cell culture and mouse models (Liang 2014, Belcher 2015). Little is known about the effect of ERRγ signaling on/in immune cells or cardiac physiology or disease (Kuiper 1997, Zaid 2015). The role of ERα and ERβ signaling has not been previously investigated in myocarditis. In Chapter 2, we showed that gonadectomy of

female BALB/c mice (in plastic caging) significantly increased acute CVB3 myocarditis and this effect was significantly reversed using estradiol pellets (**Chapter 2, Fig 2**), indicating that estrogen reduces CVB3-induced myocarditis in mice.

BPA binds ERs (Kuiper 1997, Hiroi 1999, Molina-Molina 2013, Li 2015, Zaid 2015) and so exposure to BPA has the potential to alter myocarditis. BPA binds to ERα and ERβ at a 10,000 fold lower affinity than natural estrogen, but binds ERRγ with a higher affinity (Kuiper 1997, Zaid 2015). Interestingly, BPA treatment of cultured cardiomyocytes was found to induce arrhythmias via ERβ (Belcher 2012), suggesting that detrimental effects of BPA on cardiomyocytes may be mediated via ERβ rather than ERα. In this study, we found that BPA exposure decreased the mRNA expression of ERα in the heart during CVB3 myocarditis (p=0.02) (**Fig 24A**), increased the expression of ERβ (p=0.04) (**Fig 24B**), but had no significant effect on ERRγ (p=0.38) (**Fig 24C**) and the androgen receptor (AR) (p=0.91) (**Fig 24D**). ERRγ levels were barely detectable above the housekeeping gene Hprt (i.e., RGE at 1) (**Fig 24D**), suggesting that this ER plays a minimal role in increasing inflammation following BPA exposure. Although it is not believed that BPA binds to the AR directly, one study found that BPA can act as both an agonist and antagonist for the AR (Molina-Molina 2013). Additionally, it is possible that changes in ERs may alter the expression of the AR and so the receptor was examined for completeness. Based on our finding that BPA exposure increased acute myocarditis in female BALB/c mice (**Chapter 4, Fig 2**), these data suggest that ERα signaling reduces while ERβ signaling increases myocarditis- similar to the role for these receptors in other cardiovascular diseases. These findings also suggest that BPA mediates its effect by altering ERα and ERβ expression levels in the heart and/or on cardiac inflammation.

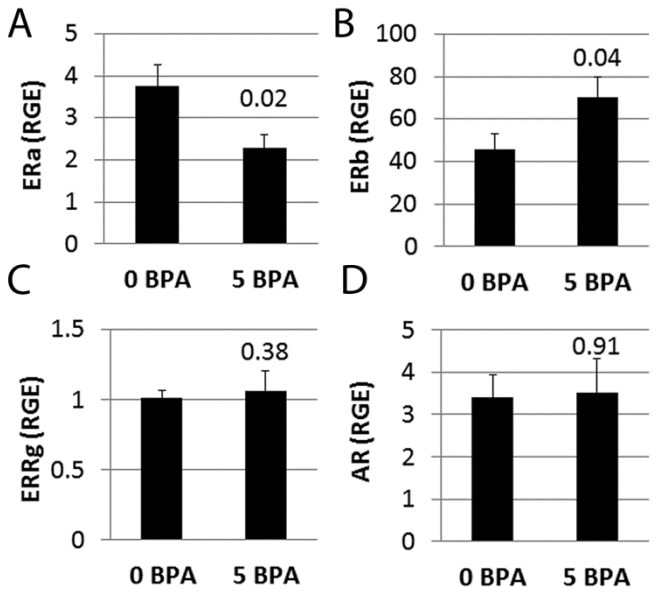


Figure 24. BPA exposure decreases ERα, but increases ERβ mRNA levels in the heart during myocarditis. Female BALB/c mice were given 0 or 5 μg/kg BW bisphenol A (0 BPA vs. 5 BPA) in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0. BPA exposure continued from day 0 to 10 pi. Hearts were harvested at day 10 pi during acute myocarditis. Relative gene expression (RGE) of **A)** ERα, **B)** ERβ, **C)** ERRγ and **D)** AR vs. the housekeeping gene HPRT were analyzed in whole hearts by qRT-PCR at day 10 pi comparing 0 to 5 μg BPA/kg BW groups. Data show the mean +/-SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 8-10 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA exposure increases activation of cardiac ERα and ERβ during myocarditis

In order to determine whether BPA exposure altered ERs at the protein level we conducted Western blots. Activation of ERs requires phosphorylation (Campesi 2016). We assessed if BPA altered activation of ERα and/or ERβ by determining protein levels of the phosphorylated form of the receptors. We found that BPA exposure did not significantly alter total protein levels of ERα (**Fig 25**) or ERβ (**Fig 26**), contrary to the mRNA results (**Fig 24**)

where we found that BPA decreased mRNA levels of ERα and increased expression of ERβ. We found that BPA exposure significantly increased protien levels of phosphorylated-ERα (p-ERα) (**Fig 25**) and phosphorylated-ERβ (p-ERβ) (**Fig 26**) in the heart of female BALB/c mice during myocarditis. These data confirm that BPA was able to activate cardiac ERα and ERβ during myocarditis. Westerns were conducted only one time using 5 mice per group and so should be repeated with larger numbers per group to strengthen these findings.

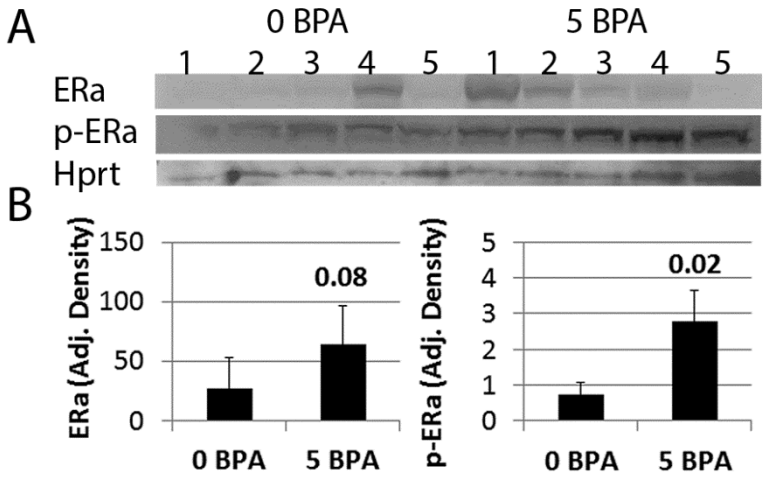


Figure 25. BPA exposure significantly increases phosphorylation of ERα (p-ERα) in the heart during myocarditis. Female BALB/c mice were given 0 or 5 μg/kg BW bisphenol A (0 BPA vs. 5 BPA) in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0. BPA exposure continued from day 0 to 10 pi. Hearts were harvested at day 10 pi during acute myocarditis. **A)** Western blot images for ERα, p-ERα and Hprt. **B)** Quantitation of western blot data for ERα (*left*) and p-ERα (*right*) adjusted against Hprt levels. Data show the mean ±SEM using a two-tailed Mann-Whitney rank test with 5 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

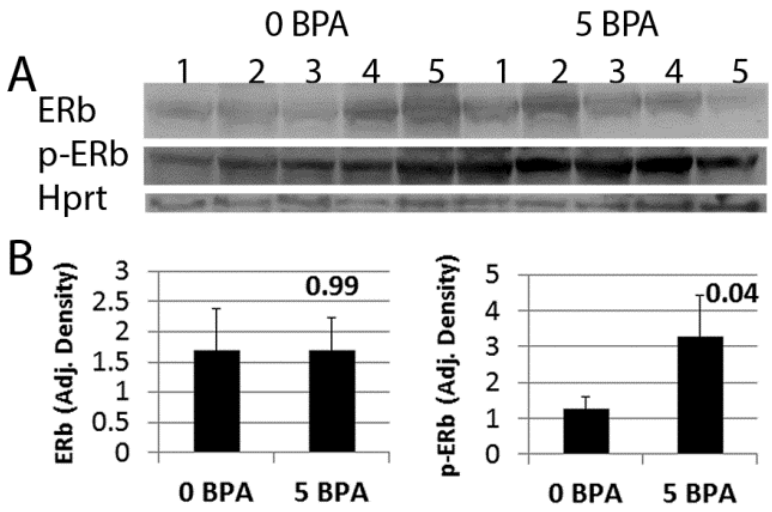


Figure 26. BPA exposure significantly increases phosphorylation of ERβ (p-ERβ) in the heart during myocarditis. Female BALB/c mice were given 0 or 5 μg/kg BW bisphenol A (0 BPA vs. 5 BPA) in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0. BPA exposure continued from day 0 to 10 pi. Hearts were harvested at day 10 pi during acute myocarditis. **A)** Western blot images for ERβ, p-ERβ and Hprt. **B)** Quantitation of western blot data for ERβ (*left*) and p-ERβ (*right*) adjusted against Hprt levels. Data show the mean ±SEM using a one-tailed Mann-Whitney rank test with 5 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA exposure increases ERα and ERβ mRNA at 24 hours pi in the spleen

To assess whether BPA exposure altered ER expression on/in immune cells during the innate immune response to CVB3 infection, we exposed mice for 2 weeks prior to ip injection with CVB3 with various doses of BPA in their drinking water and examined ERα, ERβ, ERRγ and AR expression by qRT-PCR from spleens at 24 hours pi. BPA exposure in drinking water continued until harvest. Data are shown only for the 0 and 5 μg/kg BW dose of BPA. We found that BPA exposure significantly increased ERα (p=0.04) (**Fig 27A**) and

ERβ (p=0.001) (**Fig 27B**) levels in the spleen, but had no significant effect on ERRγ (p=0.19) (**Fig 27C**) or the AR (p=0.84) (**Fig 27D**) levels compared to 0 BPA control water at 24 hours pi. Notice that ERRγ and AR levels are close to the housekeeping gene level of 1 RGE compared to the high activation level of ERα and ERβ (**Fig 27**). These data suggest that BPA exposure upregulates and/or activates ERs on/in immune cells during the innate immune response to CVB3 because the spleen is primarily composed of immune cells.

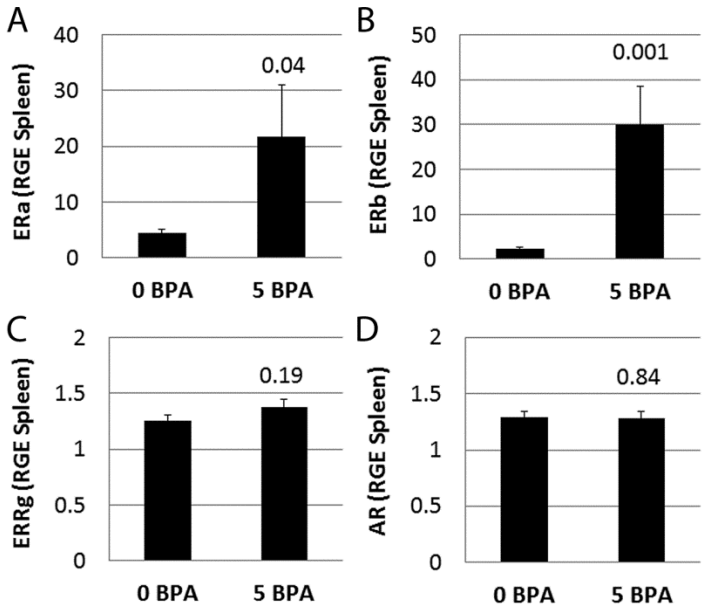


Figure 27. BPA exposure upregulates ERα and ERβ mRNA at 24 hours pi in the spleen. Female BALB/c mice were given 0 or 5 μg/kg BW bisphenol A (0 BPA vs. 5 BPA) in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0. BPA exposure continued from day 0 to 24 hours pi. Spleens were harvested at 24 hours pi during the innate immune response to CVB3. Relative gene expression (RGE) of **A)** ERα, **B)** ERβ, **C)** ERRγ and **D)** AR vs. the housekeeping gene Hprt were analyzed in whole spleens by qRT-PCR at 24 hours pi comparing 0 to 5 μg BPA/kg BW groups. Data show the mean +/-SEM using a two-tailed Mann-Whitney rank test with 8-10 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA exposure decreases VDR expression in the heart of females during myocarditis

We have found that estradiol replacement after ovariectomy increases VDR expression in the heart of females where it plays a protective role (**Chapter 3, Fig 2A**). ERα has been reported as a VDRE gene and we found that ERα is upregulated during myocarditis (**Chapter 3, Supplemental Table 1**). In Chapter 3 we showed that VDR deficient female mice had significantly increased acute myocarditis (p=0.02) (**Chapter 3, Fig 3**), indicating that VDR signaling reduces CVB3 myocarditis in female mice. Furthermore, we found that VDR deficient female mice had significantly increased T cells (**Chapter 3, Fig 4**), IFNγ and IL-17A (**Chapter 3, Fig 5**), and TLR4 pathway activation (**Chapter 3, Fig 7**) in the heart during acute CVB3 myocarditis. These are precisely the same findings we observed in WT female mice with CVB3 myocarditis exposed to BPA (**Chapter 4, Figures 2, 7B, 8, 18**). For this reason we examined the expression of the VDR and the Cyps that lead to active VitD in the heart during acute myocarditis in female mice exposed to BPA. Recall that VitD obtained from exposure to the sun or from dietary sources is hydroxylated in the liver (and other organs) by 25-hydroxylase (Cyp2R1) and carried in the bloodstream by VitD binding protein (DBP) to the kidney where it is converted by 25-OH-D-1-hydroxylase (Cyp27B1) to the active form of VitD (i.e., 1,25-dihydroxyvitamin D3), which binds the VDR (Plum 2010, Zittermann 2010, Rowling 2007, Zhu 2013). VitD is regulated in part by Cyp24A1, which actively down-regulates VitD levels (Zittermann 2010). We have found that all of these VitD-related Cyps are present in the heart during CVB3 myocarditis (**Chapter 3, Table 1**).

Here we found that BPA exposure significantly decreased VDR (p=0.02) and Cyp27B1 (p=0.01) expression in the heart during myocarditis by qRT-PCR, but had no

significant effect on Cyp2R1 (p=0.41) or Cyp24A1 (p=0.88) levels (**Fig 28**). Our findings suggest that one way that BPA could increase inflammation in the heart of female mice is by reducing VDR levels.

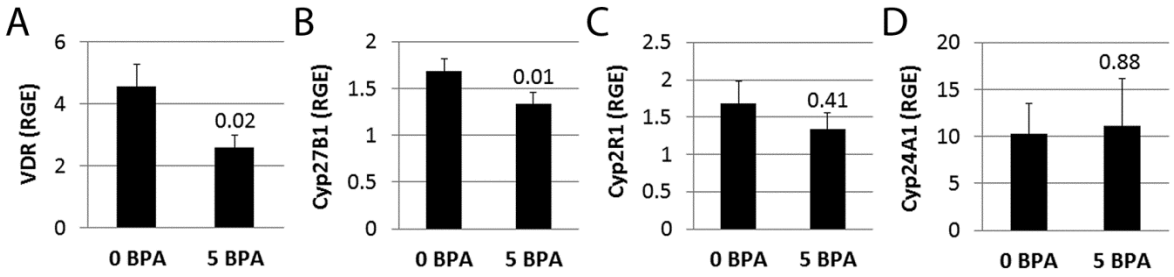


Figure 28. BPA exposure decreases VDR expression in the heart of females with myocarditis. Female BALB/c mice were given 0 or 5 μg/kg BW bisphenol A (0 BPA vs. 5 BPA) in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0. BPA exposure continued from day 0 to 10 pi. Hearts were harvested at day 10 pi during acute myocarditis. Relative gene expression (RGE) of **A)** VDR, **B)** Cyp27B1, **C)** Cyp2R1 and **D)** Cyp24A1 vs. the housekeeping gene Hprt were analyzed in whole hearts by qRT-PCR at day 10 pi comparing 0 to 5 μg BPA/kg BW groups. Data show the mean +/-SEM using a two-tailed Mann-Whitney rank test with 6-10 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA exposure did not alter protein levels of VDR in the heart during myocarditis

We used Western blots to determine whether BPA exposure altered VDR protein levels in the whole heart during myocarditis. We found that BPA exposure did not significantly alter total protien levels of VDR in the heart of female BALB/c mice with myocarditis (**Fig 29**) in contrast to our finding that VDR mRNA expression was decreased by qRT-PCR (**Fig 28**). An antibody against phosphorylated VDR was not available to check activation of the receptor. However, this Western was conducted only one time using 5 mice per group and so should be repeated with larger numbers per group to confirm these findings.

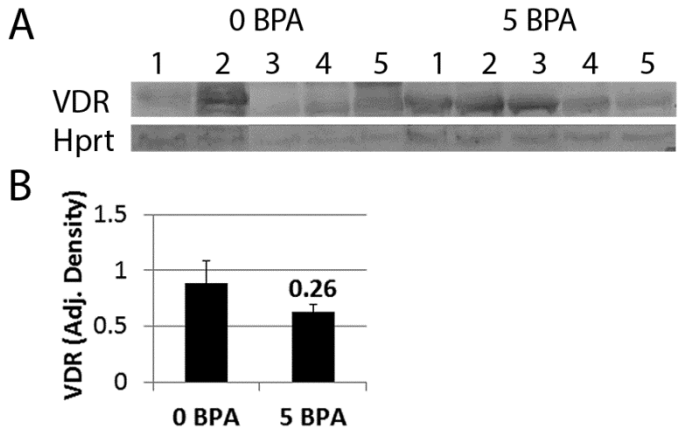


Figure 29. BPA exposure did not alter protein levels of VDR in the heart during myocarditis. Female BALB/c mice were given 0 or 5 μg/kg BW bisphenol A (0 BPA vs. 5 BPA) in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0. BPA exposure continued from day 0 to 10 pi. Hearts were harvested at day 10 pi during acute myocarditis. **A)** Western blot images for VDR and Hprt. **B)** Quantitation of western blot data for VDR adjusted against Hprt levels. Data show the mean ±SEM using a two-tailed Student's *t* test with 5 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA exposure does not alter VDR expression in the spleen at 24 hours pi

To assess whether BPA exposure altered VDR expression on/in immune cells during the innate immune response to CVB3 infection, we exposed mice for 2 weeks prior to ip injection with CVB3 with various doses of BPA in their drinking water and VDR expression by qRT-PCR from spleens at 24 hours pi. BPA exposure in drinking water continued until harvest. Data are shown only for the 0 and 5 μg/kg BW dose of BPA. We found that BPA exposure did not significantly alter VDR levels in the spleen at 24 hours pi (p=0.04) (**Fig 30**). VDR is expressed on monocyte macrophages and mast cells (Bickle 2011), which are cell types present in the spleen of BALB/c mice (Fairweather 2004B). These data suggest that the

ability of BPA to reduce VDR expression in the heart may occur on cardiac immune or tissue cells.

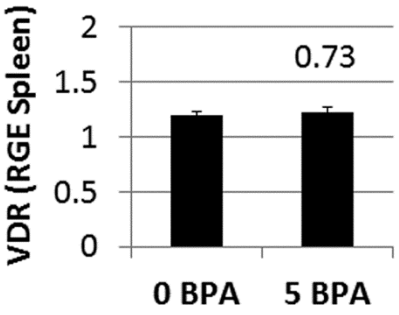


Figure 30. BPA exposure does not alter VDR expression in the spleen at 24 hours pi. Female BALB/c mice were given 0 or 5 μg/kg BW bisphenol A (0 BPA vs. 5 BPA) in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0. BPA exposure continued from day 0 to 24 hours pi. Spleens were harvested at 24 hours pi during the innate immune response to CVB3. Relative gene expression (RGE) of VDR vs. the housekeeping gene Hprt was analyzed in whole spleens by qRT-PCR at 24 hours pi comparing 0 to 5 μg BPA/kg BW groups. Data show the mean +/-SEM using a two-tailed Student's *t* test with 9-10 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

Discussion

In this study we found that BPA exposure to adult female BALB/c mice housed in plastic caging significantly increased myocarditis at day 10 pi at a high human relevant dose (25 μg BPA/L water or 5 μg BPA/kg BW) compared to control water (**Fig 2&3**). Using qRT-PCR to determine virus expression in the heart revealed that viral levels were not significantly altered by BPA exposure (**Fig 6**), suggesting that increased myocarditis was not due to BPA altering viral replication. The primary effect of BPA exposure on acute CVB3 myocarditis was to increase the number of infiltrating CD4+ T cells, according to qRT-PCR (**Fig 7B**). Interestingly, BPA exposure at all doses that we tested increased pericarditis (**Fig 4&5**) and mast cell degranulation along the pericardium (**Fig 13**), which is known to contribute to cardiac dysfunction (Afanasyeva 2004, Fairweather 2004A). When mast cells were assessed histologically using toluidine blue to detect granules we saw the primary effect of BPA on the heart was to induce degranulation of pericardial mast cells rather than myocardial or mast cells near vessels. BPA exposure significantly decreased ERα (**Fig 24**) and VDR (**Fig 28**) expression in the heart by qRT-PCR of females during myocarditis-receptors that usually protect females from myocarditis (**Chapter 3 Fig 3**). In contrast, BPA exposure significantly increased ERβ in the heart of females by qRT-PCR during myocarditis (**Fig 24**)- a receptor that has been hypothesized to be associated with worse cardiac function (Liang 2014). We found that BPA exposure activated both ERα and ERβ during myocarditis (**Fig 25&26**). Inflammatory signaling pathways known to increase myocarditis and DCM in males and that are associated with mast cell activation (i.e., TLR4, IL-1β, complement/anaphylaxis, TIMP-1) were significantly increased in females after BPA exposure (Kaya 2001, Fairweather 2006, Coronado 2012). BPA exposure in mouse models

of lupus and human immune cells have also found that BPA activates the TLR4 pathway causing the release of mature IL-1β and IL-18 (Panchanathan 2015). Thus, BPA exposure appears to have converted the cardioprotective female immune response to a proinflammatory and profibrotic immune response that is typically associated with increased myocarditis in males (**Fig 31**).

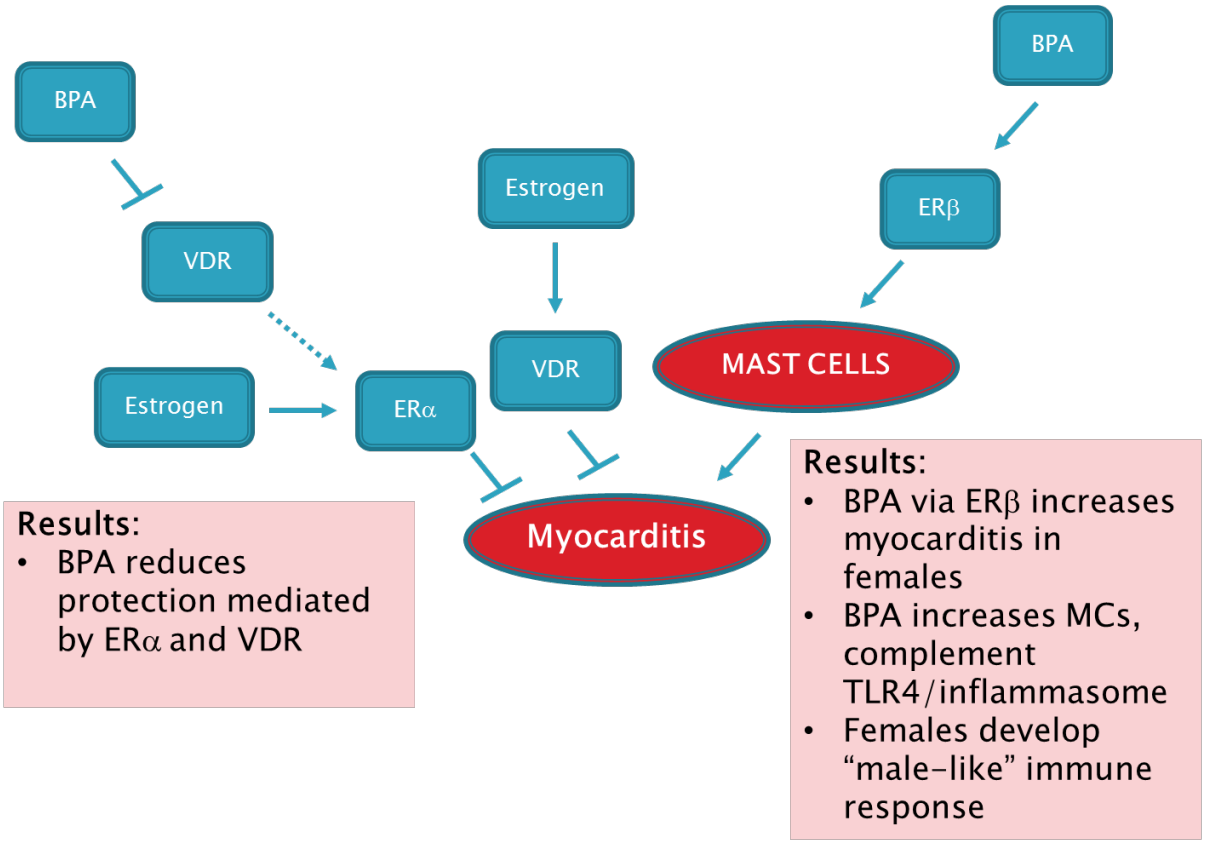


Figure 31. Possible mechanisms to explain how BPA increases myocarditis in female BALB/c mice housed in plastic cages. BPA decreases ERα expression in the heart during CVB3 myocarditis. ERα transcriptionally regulates VDR levels and causes a decrease in VDR expression in the heart. Mast cells, especially along the pericardium, are activated without the inhibitory effect of ERα and VDR to control them. Mast cells are key cells that are involved in complement and TLR4/inflammasome pathways, and without the regulating effect of ERα and VDR mast cells increase these mediators, which are usually characteristic of males. BPA also increases ERβ, which

may also lead to increased mast cells or T cells and overall increased levels of myocardial inflammation. We showed in Chapter 3 that VDR signaling protects female mice from CVB3 myocarditis. Data from this Chapter further suggests that VDR signaling in females is protective because it occurs in the context of ERα signaling. Thus, BPA exposure to females disrupts these two key signaling pathways that prevent damaging pathology after CVB3 infection.

A recent study in a rat model assessed the effect of BPA on the uterus and found that BPA treatment downregulated ERα and upregulated ERβ mRNA in a manner similar to our findings in BALB/c female mice with CVB3 myocarditis housed in plastic cages (Zaid 2015). Another study in mice found BPA increased ERβ levels which promoted cardiac arrhythmias and worse cardiac handling, while ERα was protective (Liang 2014). The opposing action of the two main ERs in these studies has been investigated and it has been found that ERα and ERβ transcriptionally cross-regulate each other (Hall 1999, La Rosa 2012, Acconcia 2015). But it is also thought that ERRγ, which BPA strongly binds to, can also regulate ERα by heterodimerizing leading to transcriptional repression (Matsushima 2007, Zaid 2015). Additionally, BPA can act as a ERβ antagonist rather than an agonist and prevent estrogen-driven non-genomic signaling pathways that could possibly be protective (Bolli 2010, Marino 2012, Acconcia 2015).

Our studies found that BPA increases myocarditis in BALB/c female mice housed in traditional plastic caging primarily by increasing CD4 T cell expression and activating pericardial mast cells. Two recent *in vitro* studies using bone marrow-derived mast cells from mice found that mast cells were activated by BPA indicating that BPA could cause mast cell degranulation (O'Brien 2014A, O'Brien 2014B). Interestingly, we noticed that BPA

exposure appeared to cause fat accumulation in the heart during acute myocarditis (data not shown). In future studies we will stain histology sections to assess lipid content. If this observation is found to be true, it would agree with epidemiological studies indicating that BPA exposure increases the risk for type II diabetes (Bodin 2014, Rezg 2014), and a study in a mouse model of atherosclerosis that found that BPA exposure increased subcutaneous and visceral adipose tissue (Fang 2014).

Here we found that BPA exposure increased IL-1β, IFNγ and IL-17 in females during myocarditis (**Fig 8**)- a cytokine profile usually associated with myocarditis in males. BALB/c females typically have elevated Th2-type immune responses with elevated IL-4, IL-33 and ST2 levels in the heart during CVB3 myocarditis (Frisancho-Kiss 2007, Coronado 2012). BPA has been found to cause shifts from a Th2 (IL-4) to Th1 (IFNγ)-type immune (Youn 2002A, Youn 2002B, Tain 2004, Kharrazian 2014, Thompson 2015), which agrees with our findings. BPA treatment in cell culture of a monocyte-like cell line derived from a leukemia patient and human peripheral blood macrophages have been found to release the proinflammatory cytokines TNFα and IL-6 and to decrease the anti-inflammatory/ regulatory cytokines IL-10 and TGFβ (Lui 2014). Lui et al showed that this effect on cytokines by BPA was mediated through ERs (both ERα and ERβ) (Lui 2014). BPA has been found to increase IFNγ in mouse bone marrow cells and isolated CD11b+ cells through via ERα (Panchanathan 2015, Youn 2002), suggesting that BPA could be acting through ERs located directly on immune cells to shift the cytokine profile in the heart.

Overall, our data in this Chapter shows that BPA at all doses studied has the potential to cause cardiac dysfunction by activating mast cells and inducing pericarditis, and that the

high human relevant dose of 25 μg BPA/L water or 5 μg BPA/kg BW significantly increases myocarditis in female mice house in traditional plastic cages.

Chapter 5

Comparison of plastic vs. glass cages in male and female BALB/c mice:

Plastic cages increase mast cells and AR expression in females, and myocarditis in males

Abstract

Myocarditis is an inflammatory heart disease that leads to DCM and heart failure in men and male mice. ERα is believed to protect women from severe myocarditis, DCM and heart failure while ERβ is thought to negatively impact cardiac function. The human population is widely exposed to the endocrine disruptor BPA from plastics, such as water bottles, plastic food containers, and receipts. Thus, BPA could increase myocarditis in females through deleterious actions of the ERβ rather than beneficial effects via ERα. Traditional plastic cages and water bottles used to house mice are believed to leach plastics into the drinking water by surface contact that could affect experiments. To our knowledge no one has examined the role of plastic caging as an exposure to an endocine disruptor in animal models such as myocarditis. In this study, we found that female BALB/c mice exposed to plastic caging alone compared to mice housed in glass cages with glass water bottles significantly increased mast cell numbers (cKit via qRT-PCR) and AR expression in the heart during CVB3 myocarditis. The same experiment in male BALB/c mice revealed that plastic caging alone increased myocarditis with elevated levels of CD11b (marker on macropahges and mast cells and other cells) and GR1 (neutrophil marker) by qRT-PCR. Plastic caging also increased genes in the heart of male BALB/c mice related to pathways known to increase myocarditis in males including complement (C4b, C5aR1), the TLR4/IL-1R (IL-1R2), remodeling genes (TIMP-1), and appeared to shift macrophages from an M1 to M2 phenotype. BPA also increased ERβ and decreased ERα and AR expression in males with myocarditis compared to glass caging. These findings suggest that traditional plastic cages and water bottles used for mouse experiments could be leaching BPA and/or other endocrine disruptors and affecting animal experiments in a sex-specific manner. Our data

suggest that the sex difference that we see in many animal models of disease may actually be driven by exposure to BPA/plastics leaching from the plastic cages used by almost every laboratory. Interestingly, the same sex differences we observe in many animal models mirror sex differences in the human population (myocarditis for example) which may indicate that the widespread exposures to plastics in our environment and food may be driving sex differences in incidence and severity of chronic inflammatory diseases in the human population.

Introduction

Dr. Ana Soto from Tufts has been working on endocrine disruptors since 1989 and is one of the leading experts. Dr. Soto was the first person to discover that laboratory supplies contain endocrine disrupting chemicals such as BPA and nonylphenol (Sonnenschein 1998). Dr. Soto first discovered that laboratory supplies contained endocrine disruptors because it was effecting her laboratory cell culture experiments and she wanted to determine what was causing the abnormality.

Recent articles have begun to address concerns associated with animal studies that are attempting to understand the role of endocrine disruptors, such as BPA, on immune function and/or disease. These articles have identified a number of potentially confounding issues including plastic cages, food and bedding containing genestein from soy or other phytoestrogens, enormous variation in the doses of BPA used in experiments, and varying exposure methods (Mirmina 2014). All of these factors could be affecting experiments that are assessing the effect of endocrine disruptors on normal physiology or disease. These factors could also be affecting all of our experiments aside from the studies examining the role of endocrine disruptors. This is a virtually unstudied area of research. Here we wanted to determine the effect of plastic cages and water bottles alone (without the addition of BPA to drinking water) on CVB3 myocarditis in female and male BALB/c mice compared to glass cages and water bottles that should not leach plastics/ BPA. Our studies were performed using soy free bedding and food to prevent additional estrogens from confounding our assessment of the effect of plastic caging on myocarditis.

Results

Plastic cages increase cKit expression (mast cells) in BALB/c females during CVB3 myocarditis

Laboratory animals are traditionally housed in polycarbonate cages with polysulfone water bottles. Both of these plastics contain BPA as one of the monomers that make up their structure, and the chemical can leach from the plastic especially when heated as occurs routinely when cages are autoclaved in order to sterilize them between uses and higher levels of plastics can be released from older cages (Howdeshell 2003). In order to determine whether the plastic cages and water bottles used in our experiments affected myocarditis, we compared glass cages and water bottles (no BPA) to plastic cages and water bottles (possible BPA or other chemicals/plastics released due to leaching). We used soy-free bedding and food to remove the effect of these estrogenic compounds from complicating the outcome. The water bottles in glass and plastic cages contained MilliQ water (i.e., did not contain additional BPA) throughout the experiment. Female BALB/c mice were infected on day 0 and myocarditis, individual immune cells, and viral levels assessed at day 10 pi, as previously.

We found that plastic cages significantly increased cKit expression (i.e., mast cell levels) in females (p=0.04) (**Fig 1**), but did not alter myocarditis/ inflammatory infiltrates (**Fig 2**) or viral levels (**Fig 3**) in the heart by qRT-PCR. These findings indicate that the plastic cages and water bottles could possibly contribute to mast cell proliferation/activation in female BALB/c mice aside from the dose of BPA added to the water in the experiments shown in **Chapter 4**. These findings will need to be confirmed using histology and/or flow cytometry to assess mast cell numbers and degranulation.

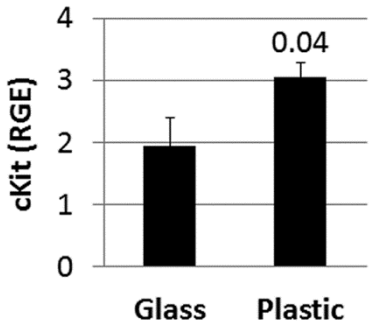


Figure 1. Plastic cages/water bottles alone increase cKit expression (i.e., mast cell numbers) in BALB/c females. Female BALB/c mice were given normal drinking water (no BPA) for 2 weeks when housed in glass cages/water bottles (0 BPA) vs. plastic cages/water bottles (possible BPA leaching) using soy-free food and bedding. Mice were injected ip with 10^3 PFU of CVB3 ip on day 0. Hearts were harvested at day 10 pi during acute myocarditis. Relative gene expression (RGE) of cKit/ CD117 (mast cells) vs. the housekeeping gene Hprt was analyzed in whole hearts by qRT-PCR at day 10 pi comparing mice in glass vs. plastic cages. Data show the mean +/-SEM using a two-tailed Student's *t* test with 9-10 mice/ group.

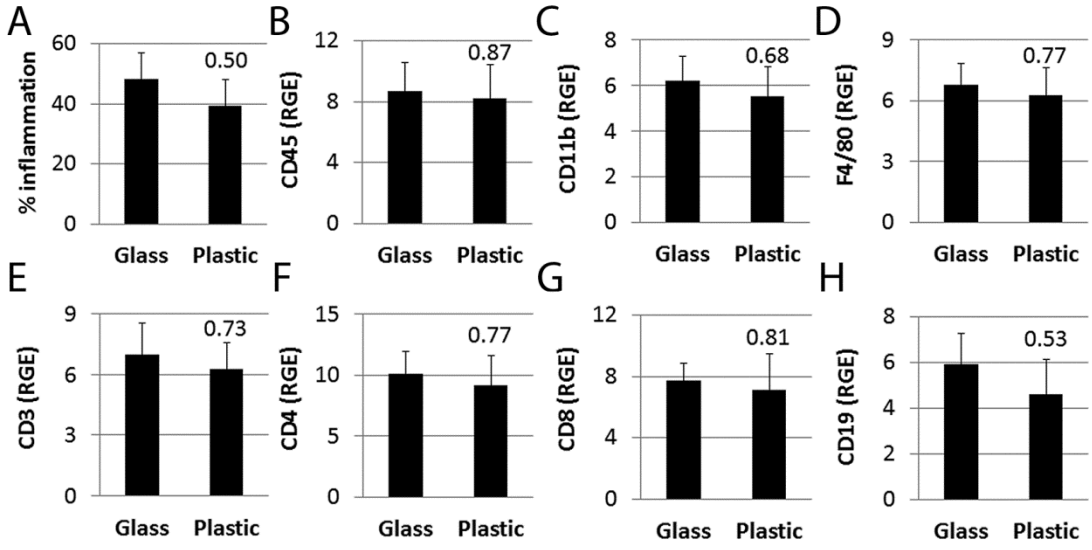


Figure 2. Plastic cages/water bottles do not alter myocardial inflammation in female BALB/c mice. Female BALB/c mice were given normal drinking water (no BPA) for 2 weeks when housed in glass cages/water bottles (0 BPA) vs. plastic cages/water bottles (possible BPA leaching) using soy-

free food and bedding. Mice were injected ip with 10^3 PFU of CVB3 ip on day 0. Hearts were harvested at day 10 pi during acute myocarditis. **A)** Myocarditis was assessed as the % inflammation compared to the size of the total heart section using H&E stained sections and a microscope grid. Relative gene expression (RGE) was used to assess **B)** total lymphocytes (CD45), **C)** CD11b+ cells (i.e., macrophages, neutrophils, mast cells), **D)** F4/80+ macrophages, **E)** all T cells (CD3), **F)** CD4+ T cells, **G)** CD8+ T cells, and **H)** CD19+ B cells compared to the housekeeping gene Hprt in whole hearts by qRT-PCR at day 10 pi comparing mice in glass vs. plastic cages. Data show the mean +/- SEM using a two-tailed Student's *t* test or the Mann-Whitney rank test with 10 mice/ group.

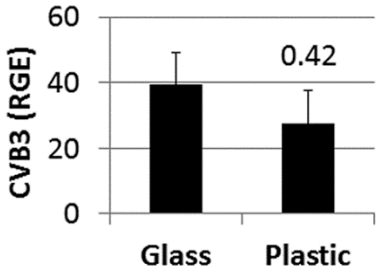


Figure 3. Viral gene expression in the heart of females was not effected by plastic cages. Female BALB/c mice were given normal drinking water (no BPA) for 2 weeks when housed in glass cages/water bottles (0 BPA) vs. plastic cages/water bottles (possible BPA leaching) using soy-free food and bedding. Mice were injected ip with 10^3 PFU of CVB3 ip on day 0. Hearts were harvested at day 10 pi during acute myocarditis. CVB3 levels in the heart were measured using qRT-PCR to determine relative gene expression (RGE) of viral genome compared to the housekeeping gene Hprt. Data show the mean +/-SEM using a two-tailed Student's *t* test or the Mann-Whitney rank test with 7-9/group.

Plastic cages increase androgen receptor expression in the hearts of female BALB/c mice during myocarditis

Since BPA is a known component of most plastic cages used in laboratory animal housing and we have found that BPA activates hormone receptors using Western blots (**Chapter 4**), we investigated whether glass vs. plastic cages altered sex hormone receptor expression in the heart of female BALB/c mice at day 10 pi with CVB3. We found that plastic cages significantly increased AR expression in females during acute myocarditis compared to glass cages using qRT-PCR (p=0.04) (**Fig 4A**), but had no significant effect on the expression of other sex hormone receptors (i.e., VDR, ERα, ERβ, and ERRγ) (**Fig 4**). Quite surprisingly we found that plastic cages and water bottles independently increased AR expression in the heart of female BALB/c mice during acute myocarditis when soy food and bedding were not present.

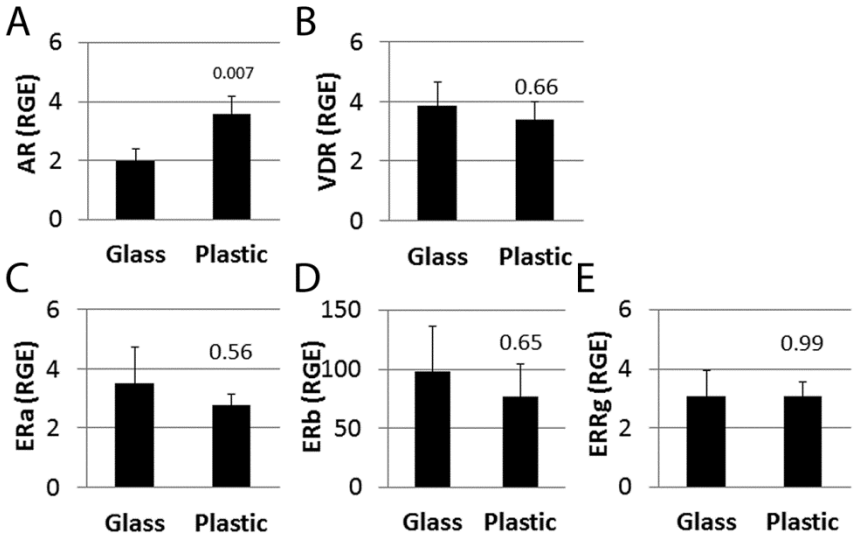


Figure 4. Plastic cages increase AR expression in the hearts of female BALB/c mice during myocarditis cages. Female BALB/c mice were given MilliQ drinking water (no BPA) for 2 weeks when housed in glass cages/water bottles (0 BPA) vs. plastic cages/water bottles (possible BPA leaching) using soy-free food and bedding. Mice were injected ip with 10^3 PFU of CVB3 ip on day 0. Hearts were harvested at day 10 pi during acute myocarditis. Relative gene expression (RGE) vs. Hprt controls for **A)** AR, **B)** VDR, **C)** ERα, **D)** ERβ, and **E)** ERRγ mRNA by qRT-PCR at day 10 pi

comparing glass to plastic cages. Data show the mean ±SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 10 mice/ group.

Plastic cages increase myocarditis in male BALB/c mice during CVB3 myocarditis

In this study, we compared glass cages and water bottles to traditional plastic cages and water bottles during myocarditis in male BALB/c mice using control water (containing no BPA) to assess the effect of caging on myocarditis. Mice received soy-free food and bedding. We found that plastic cages significantly increased myocarditis in males compared to glass cages assessed histologically (p=0.01) (**Fig 5**). Male BALB/c mice in our model of CVB3 myocarditis typically have inflammation ranging from 30-50% of the heart section, and these data were obtained with traditional plastic caging (Coronado 2012). These data suggest that the plastic cages used to house mice have been increasing myocarditis in male BALB/c mice, creating a "sex-difference" in myocarditis and DCM. Plastic cages (no additional BPA) increased cKit expression (i.e., mast cells) but not myocardial inflammation in BALB/c females (**Fig 1 and 2**). Interestingly, the same sex difference that we observe for myocarditis and DCM in BALB/c mice housed in plastic cages (i.e., worse in males) exists for myocarditis and DCM patients (McNamara 2011, Fairweather 2013). Because we found that plastic cages are affecting myocarditis in males (aside from BPA exposure), in future studies we will examine the effect of BPA exposure on myocarditis in male mice housed in plastic cages.

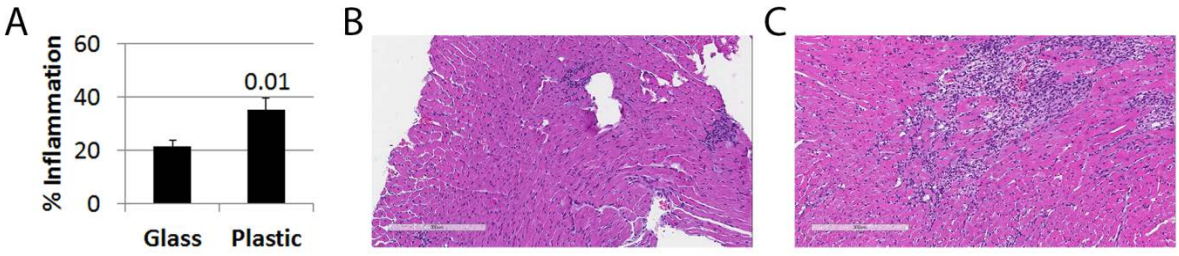


Figure 5. Plastic cages increase myocarditis in male mice. Male BALB/c mice were given normal drinking water (no BPA) for 2 weeks and housed either in glass cages/water bottles (0 BPA) vs. plastic cages/water bottles (possible BPA leaching) using soy-free food and bedding. Mice were injected ip with 10^3 PFU of CVB3 ip on day 0. Hearts were harvested at day 10 pi during acute myocarditis. **A)** Myocarditis was assessed as the % inflammation compared to the size of the total heart section using H&E stained sections and a microscope grid comparing mice in glass vs. plastic cages. Data show the mean +/-SEM using a two-tailed Student's *t* test with 18-20 mice/ group. Representative photos of heart sections stained with H&E for **B)** glass and **C)** plastic housed mice. The grey bar represents 300μm.

Plastic cages increase GR1, CD11b and CD14, but decrease cKit (mast cells) in males during myocarditis

Comparing traditional plastic cages and water bottles to glass cages and water bottles (soy-free food and bedding and no BPA added to drinking water), we found that housing male BALB/c mice in plastic cages significantly increased expression of GR1 (p=0.01), CD11b (p=0.0007), and CD14 (p=0.003), but significantly decreased cKit (p=0.00003)- a marker of mast cells (**Fig 6**) using qRT-PCR. Plastic cages did not significantly alter CD45 (p=0.42) or F4/80 (p=0.56) expression in the heart of males with myocarditis compared to glass cages (**Fig 6**). These data suggest that the dominant increase in CD11b+ cells in males with myocarditis is mediated at least in part by exposure to plastics/ chemicals that leach from plastic cages and water bottles.

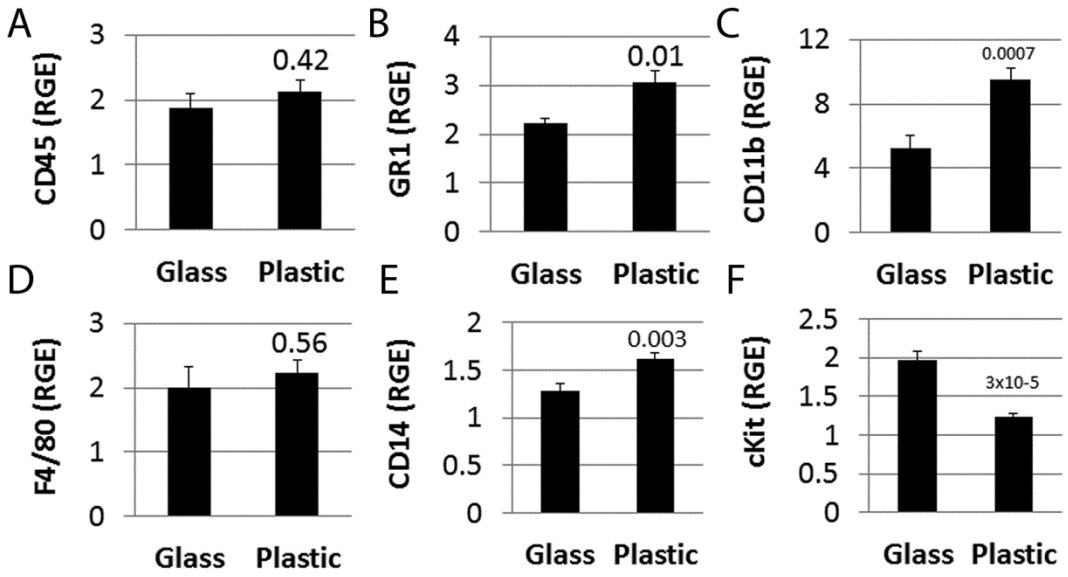


Figure 6. Plastic cages increase GR1, CD11b and CD14 expression in the heart during myocarditis. Male BALB/c mice were given normal drinking water (no BPA) for 2 weeks and housed either in glass cages/water bottles (0 BPA) vs. plastic cages/water bottles (possible BPA leaching) using soy-free food and bedding. Mice were injected ip with 10^3 PFU of CVB3 ip on day 0. Hearts were harvested at day 10 pi during acute myocarditis. Relative gene expression (RGE) was used to assess **A)** CD45 (total lymphocytes), **B)** GR1 (neutrophils), C**)** CD11b+ cells (i.e., macrophages, neutrophils, mast cells), **D)** F4/80+ macrophages, **E)** CD14 (part of TLR4 signaling complex on macrophages and mast cells), and **F)** cKit (mast cells and stem cells) compared to the housekeeping gene Hprt in whole hearts by qRT-PCR at day 10 pi comparing mice in glass vs. plastic cages. Data show the mean +/-SEM using a two-tailed Student's *t* test with 10 mice/ group.

Plastic cages have no significant effect on T or B cell markers in males during myocarditis

Comparing traditional plastic cages and water bottles to glass cages and water bottles (soy-free food and bedding and no BPA added to drinking water), we found that housing male BALB/c mice in plastic cages had no significant effect on CD3 (p=0.14), CD4 (p=0.97), CD8 (p=0.33), CD19 (p=0.34) or Foxp3 (p=0.41) expression compared to mice

housed in glass cages using qRT-PCR (**Fig 7**). We have shown previously that T cells do not play a major role in sex differences in myocarditis in BALB/c mice (Frisancho-Kiss 2009, Coronado 2012). These data further confirm that the elevated myocarditis observed in male BALB/c mice is mediated at least in part by being housed in plastic cages.

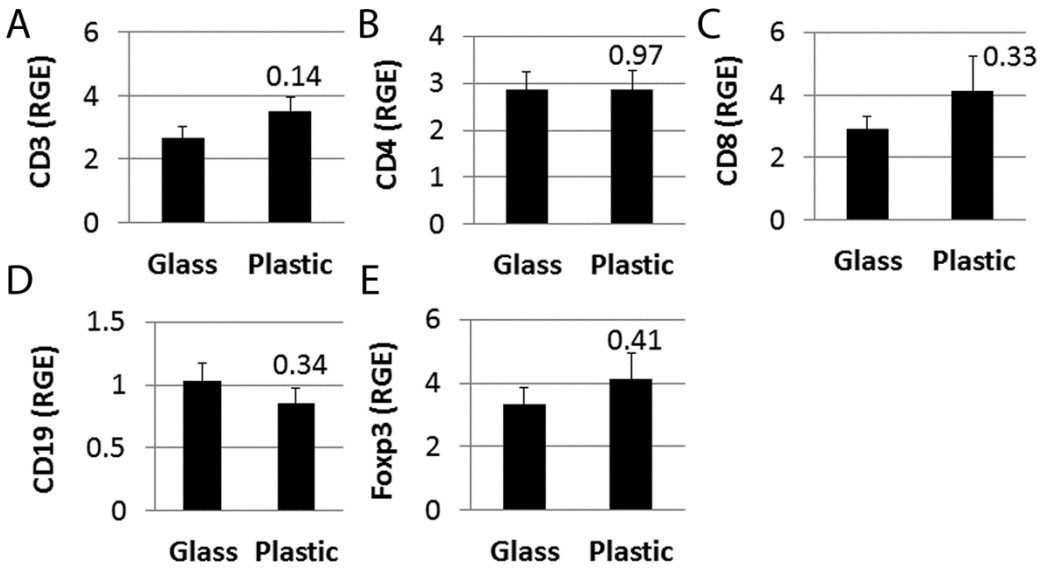


Figure 7. Plastic cages have no significant effect on T or B cell markers in males during myocarditis. Male BALB/c mice were given normal drinking water (no BPA) for 2 weeks and housed either in glass cages/water bottles (0 BPA) vs. plastic cages/water bottles (possible BPA leaching) using soy-free food and bedding. Mice were injected ip with 10^3 PFU of CVB3 ip on day 0. Hearts were harvested at day 10 pi during acute myocarditis. Relative gene expression (RGE) was used to assess **A)** CD3 (total T cells), **B)** CD4, **C)** CD8, **D)** CD19 (as a marker for B cells), and **E)** Foxp3 (Treg) compared to the housekeeping gene Hprt in whole hearts by qRT-PCR at day 10 pi comparing mice in glass vs. plastic cages. Data show the mean +/-SEM using a two-tailed Student's *t* test with 10 mice/ group.

Plastic cages have no significant effect on CVB3 gene expression in males during myocarditis

Comparing traditional plastic cages and water bottles to glass cages and water bottles (soy-free food and bedding and no BPA added to drinking water), we found that housing male BALB/c mice in plastic cages had no significant effect on CVB3 genome expression in the heart by qRT-PCR (p=0.40). These data indicate that the increase in myocarditis found in male BALB/c mice in plastic compared to glass cages is not due to increased virus levels in the heart. Additionally, we do not see a sex difference in viral replication when mice are housed in plastic cages (Fairweather 2001, Frisancho-Kiss 2007, Coronado 2012).

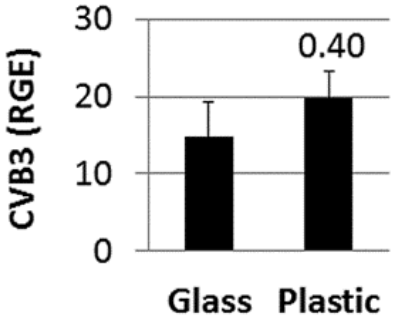


Figure 8. Viral gene expression in the heart of males was not effected by plastic cages. Male BALB/c mice were given normal drinking water (no BPA) for 2 weeks and housed either in glass cages/water bottles (0 BPA) vs. plastic cages/water bottles (possible BPA leaching) using soy-free food and bedding. Mice were injected ip with 10^3 PFU of CVB3 ip on day 0. Hearts were harvested at day 10 pi during acute myocarditis. CVB3 levels in the heart were measured using qRT-PCR to determine relative gene expression (RGE) of viral genome compared to the housekeeping gene Hprt. Data show the mean +/-SEM using a two-tailed Student's *t* test with 10 mice/ group.

Plastic cages shift the M1/ M2 profile in males during myocarditis (no BPA added to water)

Comparing traditional plastic cages and water bottles to glass cages and water bottles (soy-free food and bedding and no BPA added to drinking water), we found that housing

male BALB/c mice in plastic cages significantly decreased the M1 marker Cxcl10 *(p*=0.04) and significantly increased the M2 marker Ym1 (p=0.04) (**Fig 9**) using qRT-PCR, suggesting a shift to a more Th2-type immune response in the macrophage population. We showed previously that the dominant CD11b+ population in males during myocarditis has characteristics of M2 or myeloid-derived suppressor cells (MDSCs) as well as expressing TLR4 (Frisancho-Kiss 2007, Frisancho-Kiss 2009, Abston 2012B, Coronado 2012, Fairweather 2015). These findings suggest that the plastic cages that males are housed in are contributing to this phenotype during myocarditis. In future experiments we will examine cytokine profiles in the heart in plastic vs. glass cages in male mice with myocarditis.

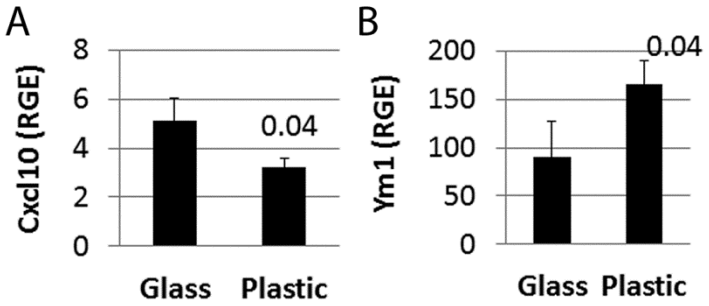


Figure 9. Plastic cages shift the M1/M2 profile in males during myocarditis. Male BALB/c mice were given normal drinking water (no BPA) for 2 weeks and housed either in glass cages/water bottles (0 BPA) vs. plastic cages/water bottles (possible BPA leaching) using soy-free food and bedding. Mice were injected ip with 10^3 PFU of CVB3 ip on day 0. Hearts were harvested at day 10 pi during acute myocarditis. Relative gene expression (RGE) was used to assess **A)** Cxcl10 (a marker of M1 macrophages) and **B)** Ym1 (a marker of M2 macrophages) compared to the housekeeping gene Hprt in whole hearts by qRT-PCR at day 10 pi comparing mice in glass vs. plastic cages. Data show the mean +/-SEM using a one-tailed Student's *t* test with 10 mice/ group.

Plastic cages increase fibrosis/remodeling-associated genes in the heart of males during myocarditis

We have reported previously that increased complement activation, TLR4/IL-1R family members (i.e., inflammasome-related genes), and Timp1 in male BALB/c mice during acute myocarditis leads to increased remodeling, fibrosis and DCM during the chronic stage of myocarditis (Fairweather 2006, Coronado 2012, Abston 2012B). Comparing traditional plastic cages and water bottles to glass cages and water bottles (soy-free food and bedding and no BPA added to drinking water), we found that housing male BALB/c mice in plastic cages significantly increased cardiac expression of C4b (p=0.02), C5aR1 (p=0.003), IL1R2 (p=0.0005), and Timp1 (p=0.01) during myocarditis compared to glass cages using qRT-PCR (**Fig 10**). In future experiments we will examine more complement, inflammasome and remodeling genes in males with myocarditis that have been housed in plastic cages compared to glass cages, and assess whether males develop fibrosis during acute myocarditis. These data indicate that plastics/ chemicals that leach from plastic cages and water bottles contribute to the characteristic proinflammatory and profibrotic profile in male BALB/c mice with myocarditis.

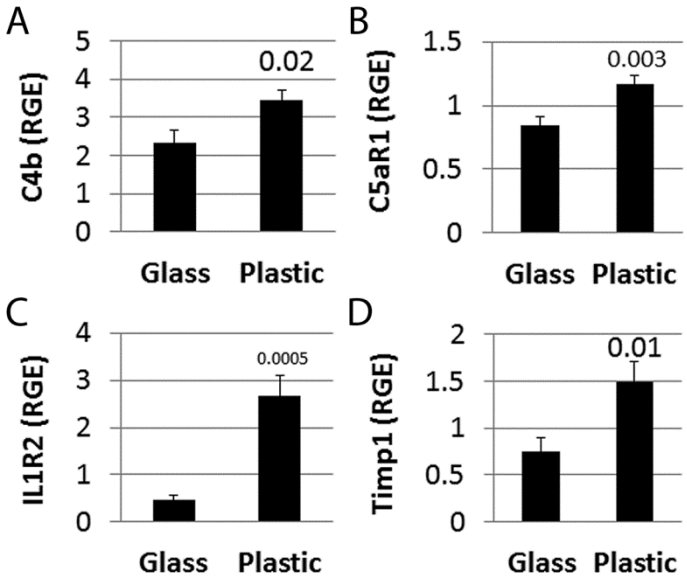


Figure 10. Plastic cages increase fibrosis/remodeling-associated genes in the heart of males during myocarditis. Male BALB/c mice were given normal drinking water (no BPA) for 2 weeks and housed either in glass cages/water bottles (0 BPA) vs. plastic cages/water bottles (possible BPA leaching) using soy-free food and bedding. Mice were injected ip with 10^3 PFU of CVB3 ip on day 0. Hearts were harvested at day 10 pi during acute myocarditis. Relative gene expression (RGE) was used to assess **A)** C4b, **B)** C5aR1, **C)** IL1R2, and **D)** Timp1 compared to the housekeeping gene Hprt in whole hearts by qRT-PCR at day 10 pi comparing mice in glass vs. plastic cages. Data show the mean +/-SEM using a two-tailed Student's *t* test with 10 mice/ group.

Plastic cages decrease ERα and AR expression in the heart of males during myocarditis, but increase ERβ

Comparing traditional plastic cages and water bottles to glass cages and water bottles (soy-free food and bedding and no BPA added to drinking water), we found that housing male BALB/c mice in plastic cages significantly decreased cardiac expression of ERα (p=0.007) and AR (p=0.0009), but significantly increased expression of ERβ (p=0.02) from

whole hearts by qRT-PCR (**Fig 11**). Plastic cages had no significant effect on ERRγ expression in the heart of males with myocarditis compared to glass cages using qRT-PCR (p=0.10) (**Fig 11D**). These data indicate that plastic cages and water bottles alone are significantly altering sex hormone receptor expression in the heart during myocarditis in BALB/c males. This change in the ratio of ERs and ARs could contribute to sex differences in myocarditis.

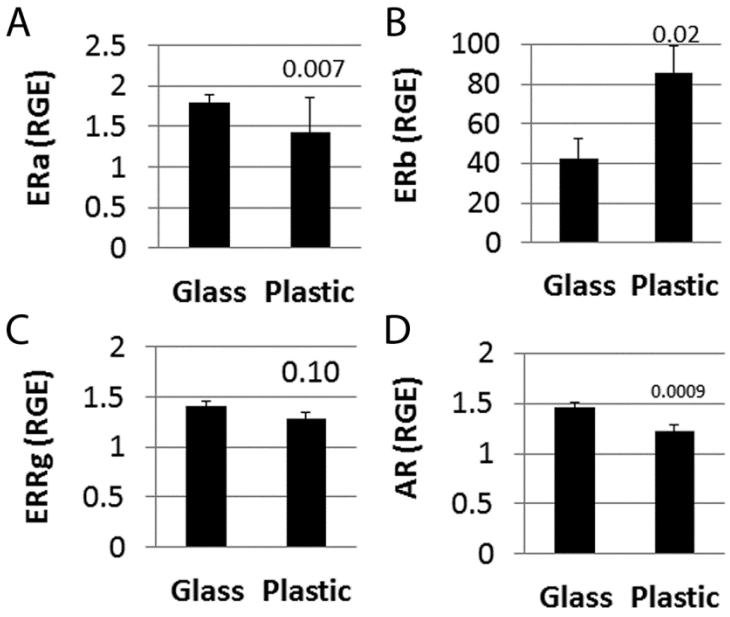


Figure 11. Plastic cages decrease ERα and AR expression in the heart of males during myocarditis, but increase ERβ. Male BALB/c mice were given normal drinking water (no BPA) for 2 weeks and housed either in glass cages/water bottles (0 BPA) vs. plastic cages/water bottles (possible BPA leaching) using soy-free food and bedding. Mice were injected ip with 10^3 PFU of CVB3 ip on day 0. Hearts were harvested at day 10 pi during acute myocarditis. Relative gene expression (RGE) vs. Hprt controls of **A)** ERα, **B)** ERβ, **C)** ERRγ, and **D)** AR mRNA by qRT-PCR at day 10 pi comparing glass caging to plastic caging. Data show the mean ±SEM using a two-tailed Student's *t* test with 10 mice/ group.

Plastic cages increase VDR expression in the heart of males during myocarditis

Comparing traditional plastic cages and water bottles to glass cages and water bottles (soy-free food and bedding and no BPA added to drinking water), we found that housing male BALB/c mice in plastic cages significantly increased VDR expression in the heart during myocarditis by qRT-PCR (p=0.001) (**Fig 12**). We showed in Chapter 3 that VDR increased myocarditis in male mice (**Chapter 3, Fig 3**). These data indicate that plastics/ chemicals that leach from plastic cages lead to increased expression of VDR in the heart of males during CVB3 myocarditis, contributing to the sex differences in disease in this model.

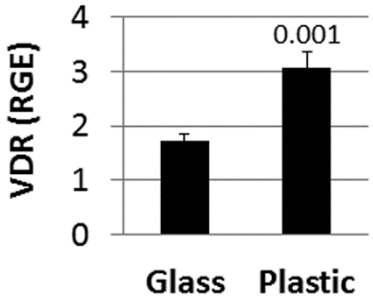


Figure 12. Plastic cages increase VDR expression in the heart of males during myocarditis. Male BALB/c mice were given normal drinking water (no BPA) for 2 weeks and housed either in glass cages/water bottles (0 BPA) vs. plastic cages/water bottles (possible BPA leaching) using soy-free food and bedding. Mice were injected ip with 10^3 PFU of CVB3 ip on day 0. Hearts were harvested at day 10 pi during acute myocarditis. Relative gene expression (RGE) vs. Hprt controls of VDR mRNA by qRT-PCR at day 10 pi comparing glass caging to plastic caging. Data show the mean ±SEM using a two-tailed Student's *t* test with 10 mice/ group.

Discussion

In **Chapter 4**, **Figure 31** we showed how BPA exposure in the drinking water of female BALB/c mice allowed increased myocarditis by decreasing ERα and VDR in the heart. In this Chapter we show the same Figure and now highlight how plastic cages and water bottles themselves (presumably plastics/ BPA leached from the cages) are able to increase AR and cKit (mast cells) expression in female BALB/c mice, which are factors known to increase myocarditis (**Fig 13**). The overall myocarditis score in females was probably not significantly altered by plastic cages because the caging did not significantly alter ERs, the VDR or T cells. Thus, the caging does not appear to affect female BALB/c mice as strongly as it does male mice.

Figure 13. Possible mechanisms to explain how plastic cages increase myocarditis in female BALB/c mice. In this Chapter we found that the plastic cages that the female BALB/c mice were housed in caused an increase in AR and cKit expression (i.e., mast cells) assessed by qRT-PCR in the heart during CVB3 myocarditis. Elevated AR and mast cells in females are likely to increase myocarditis.

We have shown that male BALB/c mice have increased CVB3 myocarditis due to a testosterone-mediated increase in CD11b+ macrophages and mast cells compared to females (Frisancho-Kiss 2009, Coronado 2012, Fairweather 2015). However, testosterone was only able to partially mediate the sex difference (i.e., gonadectomy did not prevent myocarditis). Surprisingly, housing male BALB/c mice in plastic cages had a significant effect on CVB3

myocarditis (**Fig 5**). In males we found that plastic cages and water bottles significantly increased myocarditis at day 10 pi compared to glass cages with a large increase in CD11b expression in the heart measured by qRT-PCR (**Fig 6C**). Previously when male BALB/c mice underwent gonadectomy to remove circulating testosterone levels we found that the only inflammatory cells significantly affected during CVB3 myocarditis were those expressing CD11b (Frisancho-Kiss 2009). Additionally, the dominant cell population in the heart during myocarditis in male BALB/c mice and myocarditis biopsies from patients is CD11b+ cells (Fairweather 2014). We have found that CD11b+ macrophages and mast cells in male BALB/c mice express TLR4 and IL-1β and are involved in promoting remodeling in males that leads to DCM and heart failure (Fairweather 2003, Fairweather 2004B, Frisancho-Kiss 2009, Onyimba 2011, Coronado 2012, Fairweather 2014). Recall that CD11b is also called CR3. A proteomic study of sera from myocarditis patients that examined the factors during acute myocarditis that predicted progression from myocarditis to DCM found that complement pathways were primarily responsible (Cooper 2010). When we examined the data from that study by sex we found that the top gene predicting progression from myocarditis to DCM in men with myocarditis was VitD binding protein, which works with complement pathways to activate mast cells and macrophages and TLR4/ inflammasome pathways (Rook 1986, Wang 2004, Baroni 2007, Liu 2006, Liu 2007, Christakos 2016). In Chapter 3 we showed that the VDR increases myocarditis in male mice, and here we found that plastic cages increased VDR expression in the heart of male BALB/c mice by qRT-PCR (**Fig 12**). Additionally, plastic cages decreased ERα and increased ERβ in male BALB/c mice during CVB3 myocarditis (**Fig 11**), which is likely to enhance disease in males. Both males and females express ERs and the AR and it is likely that the ratio of these receptors is

key in how inflammation is regulated by sex hormones. Additionally, plastic cages appear to shift macrophages from a M1 to M2-type phenotype in males with myocarditis. This mixed Th1/Th2, M2b-type response (M2b macrophages express TLR4 and release IL-1β and are highly profibrotic as well as being proinflammatory) is characteristic of our analysis of the type of immune cells that allow the damaging remodeling in male mice with myocarditis that leads to DCM (Fairweather 2006, Coronado 2012, Fairweather 2012A).

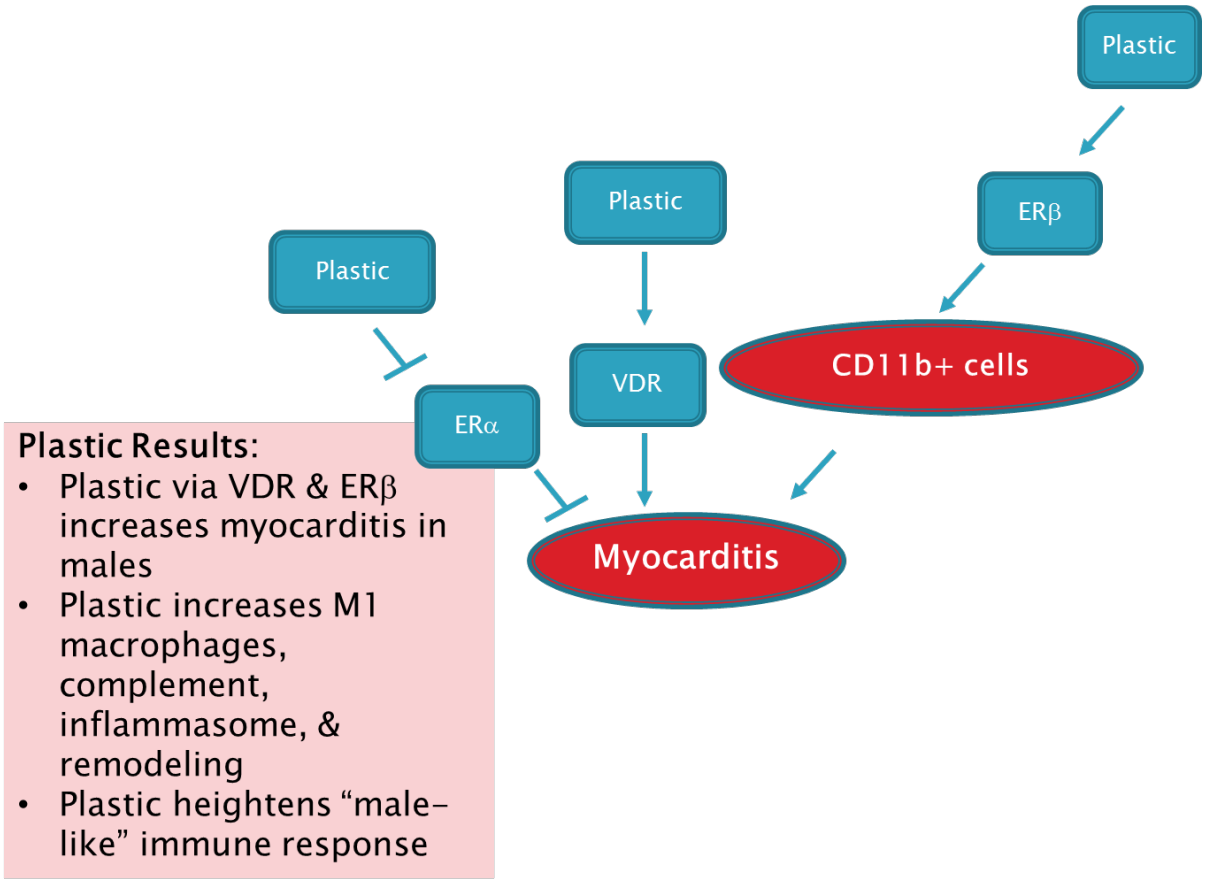


Figure 14. Possible mechanisms to explain how plastic cages increase myocarditis in male BALB/c mice. In this Chapter we found that the plastic cages that the male BALB/c mice were housed in caused an increase in myocarditis and immune cell populations assessed by qRT-PCR in the heart during CVB3 myocarditis.

We do not observe a sex difference in viral replication in the heart of BALB/c male and female mice (Frisancho-Kiss 2007, Frisancho-Kiss 2009, Coronado 2012). Here we found that plastic cages had no significant effect on viral levels in the heart during myocarditis in male or female mice by qRT-PCR (**Fig 3&8**) and plaque assay (data not shown). These data further suggest that the cages are mediating the sex difference in CVB3 myocarditis in BALB/c mice by amplifying a damaging type of inflammation that has no direct relationship to viral replication in the heart. Overall, this data suggests that there is an association between plastic exposure from cages and water bottles and sex differences in myocarditis.

Chapter 6

BPA exposure decreases myocarditis in female BALB/c mice housed in glass cages

Abstract

Myocarditis is an inflammatory heart disease that leads to DCM and heart failure. Sex hormones play a vital role in development of myocarditis with testosterone driving disease in males whereas, estrogen mediates cardioprotection in females. Since myocarditis is influenced by sex hormones, it is highly probable that endocrine disruptors, which interfere with natural hormones and ERs in particular, could play a part in the progression of the disease. The human population is exposed to the endocrine disruptor BPA from plastics like water bottles, plastic food containers, and receipts, for example. To our knowledge no one has examined the role of endocrine disruptors like BPA on myocarditis. Glass cages and soy-free food and bedding were used in these experiments in order to assess the effect of BPA on female BALB/c mice without the confounding effect of plastics, BPA or other chemicals leaching from plastic caging. We found that all doses of BPA decreased myocarditis in female BALB/c mice compared to water controls, and we chose the high human relevant dose (25 μg/L or 5 μg BPA/kg BW) of BPA for continued analysis. BPA exposure decreased expression of all CD11b+ populations including GR1, F4/80, CD14 and cKit (mast cells), but had no significant effect on T cells. Interestingly, BPA exposure significantly decreased viral RNA levels and viral replication in the heart even though it decreased IFNγ (which can decrease virus) and increased IL-4. Decreased IFNγ may be the result of BPA strongly inhibiting the TLR4 signaling, which is able to increase IFNs. BPA significantly decreased ERα expression in the heart of females in glass cages, but had no significant effect on VDR or other hormone expression. BPA exposure for the same amount of time as in other experiments had no significant effect on ER expression in the heart of uninfected female BALB/c mice suggesting co-exposure (i.e., BPA exposure plus viral infection) is important

in mediating the effect of BPA. These data show that BPA exposure that is carefully controlled for (i.e., no other sources of BPA to confound the results) by using glass cages and water bottles and soy-free bedding and food is able to decrease CVB3 myocarditis in females. Because populations are likely to be exposed to BPA and other plastics from a wide variety of sources (i.e., food, water, receipts, photocopy paper, etc.) and exposure routes (i.e., oral, cutaneous, inhalation) future experiments will need to examine the effect of BPA exposure from multiple sources on inflammatory diseases like myocarditis.

Introduction

Recent articles have begun to address concerns associated with animal studies that are attempting to understand the role of endocrine disruptors, such as BPA, on immune function and/or disease. These articles have brought up a number of potentially confounding issues including plastic cages, food and bedding containing the phytoestrogen soy or other phytoestrogens, enormous variation in the doses of BPA used, and varying exposure methods (Mirmina 2014). All of these factors could be affecting experiments that are assessing the effect of endocrine disruptors on normal physiology or disease. In **Chapter 5** we found that plastic cages and water bottles (without the addition of BPA in the drinking water) significantly increased myocarditis in male BALB/c mice, and increased cKit (mast cells) and AR expression in the heart of female BALB/c mice with myocarditis. These data were not influenced by phytoestrogens, because the experiments were performed with soy-free food and bedding. These findings indicate that caging is indeed affecting our experiments in a sex-specific manner.

Here we wanted to determine the effect of BPA exposure on female BALB/c mice without the potentially confounding effect of the presence of plastic cages and water bottles. In order to do this we used glass cages and water bottles as well as soy-free food and bedding. We exposed mice to BPA at various concentrations, as before, for 2 weeks and then examined myocarditis at day 10 pi. BPA exposure was continued from day 0 to day 10 pi, as previously. We have not found any published reports of investigators examining the effect of BPA on normal physiology or disease using glass cages and water bottles to prevent plastics potentially leaching from the cages and affecting their results.

Results

BPA decreases CVB3 myocarditis in female BALB/c mice housed in glass cages

In **Chapter 4** we showed that BPA exposure of female BALB/c mice in standard plastic cages/water bottles significantly increased myocarditis (**Chapter 4, Fig 2**). Additionally, we found that plastic cages and water bottles in themselves (e.i., no BPA added to water) increased cKit expression/ mast cells (**Chapter 5, Fig 1**) and AR expression in the heart of females with myocarditis (**Chapter 5, Fig 4**). In this chapter we wanted to determine whether the same result would occur if glass cages and water bottles were used instead of plastic cages to remove any possibility of plastic products leaching out and altering the dose of BPA. To assess the effect of BPA exposure on CVB3 myocarditis in glass cages with glass water bottles, 6-8 week old female BALB/c mice were given varying doses of BPA in their drinking water for two weeks prior to infection with CVB3 ip on day 0, as previously. Mice in all of the experiments in this Chapter were housed in glass cages using glass water bottles and were provided bedding and food free of soy, an estrogenic agent. As a positive control, we also included a set of mice in plastic cages/water bottles with 0 BPA, but we have not shown the data in the figures in this Chapter. The doses of BPA that were administered were the same as in **Chapter 4** and included 2.5, 25, and 250 μg BPA/L in drinking water, which is equivalent to an estimated intake of 0.5, 5, and 50 μg BPA/kg BW, respectively. Three doses were used in order to determine whether the concentration of BPA altered myocarditis when mice were housed in glass cages instead of plastic cages (i.e., is there a dose effect for BPA?). BPA treatment was continued from day 0 after infection until harvest at day 10 pi. Myocarditis was assessed histologically at day 10 pi, during acute myocarditis, by determining the % inflammation in heart sections corrected for the size of the heart.

We found that BPA exposure of mice housed in glass cages/ water bottles significantly decreased myocarditis (p=0.0001). Controlling for multiple comparisons revealed that the 2.5 μg BPA/L water dose which is equivalent to an estimated intake of 0.5 μg BPA/kg BW, the 25 μg BPA/L water dose which is equivalent to an estimated intake of 5 μg BPA/kg BW, and the 250 μg BPA/L water dose which is equivalent to an estimated intake of 50 μg BPA/kg BW, significantly decreased myocarditis at day 10 pi compared to control water containing no BPA when mice were housed in glass cages (p<0.001) (**Fig 1**). Representative photos of the effect of BPA exposure on CVB3 myocarditis using glass cages and water bottles are shown in **Figure 2**. These data further indicate that chemicals/ plastics released from plastic cages and water bottles alter myocarditis in female BALB/c mice. Regardless of dose, BPA exposure significantly reduced myocarditis in female mice if they were housed in glass cages/water bottles. Thus, the lowest concentration of BPA that we tested significantly decreased myocarditis. Additionally, there were no significant differences between doses indicating that there was no dose effect of BPA exposure.

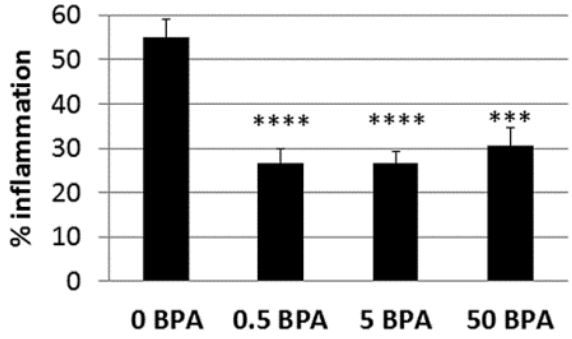


Figure 1. BPA decreases myocarditis in BALB/c female mice housed in glass cages with glass water bottles. Female BALB/c mice were given increasing doses of BPA in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 inflammation examined at day 10 pi, during peak myocarditis. Soy-free bedding and food and glass cages and water bottles were used.

BPA exposure was continued from day 0 to harvest at day 10 pi. Myocarditis was assessed as the % inflammation in the heart H&E compared to the overall size of the heart section by histology using an eyepiece grid. Estimated BPA intakes were determined as follows: 0 (0 BPA), 0.5 (0.5 BPA), 5 (5 BPA), and 50 (50 BPA) µg BPA/kg BW were obtained by dosing water with 0, 2.5, 25 or 250 µg BPA/L, respectively. Data show the mean +/-SEM, n=18-37/group. One-way ANOVA found a significant difference existed between groups (p=0.004). After controlling for multiple comparisons, the 0.5 µg BPA/kg BW and 5 µg BPA/kg BW doses were significantly decreased compared to control water using post-hoc Student's t tests (****, p<0.0001) as well as 50 µg BPA/kg BW dose (***, p<0.001).

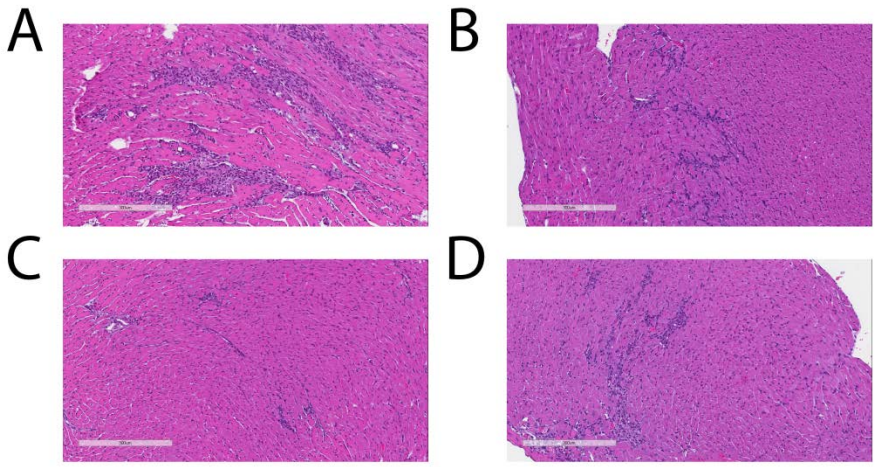


Figure 2. Representative photos of the effect of BPA exposure on CVB3 myocarditis in female mice housed in glass cages. Female BALB/c mice were given increasing doses of BPA in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 inflammation examined at day 10 pi, during peak myocarditis. Soy-free bedding and food and glass cages and water bottles were used. BPA exposure was continued from day 0 to harvest at day 10 pi. Estimated BPA intakes were determined as follows: 0, 0.5, 5, and 50 µg BPA/kg BW were obtained by dosing water with 0, 2.5, 25 or 250 µg BPA/L, respectively. Inflammation in the heart was assessed using H&E stain where

cardiac tissue appears pink and inflammation dark purple. Representative photos depict **A)** 0, **B)** 0.5, **C)** 5, and **D)** 50 μg BPA/kg BW. The grey bar is 300μm in length.

BPA significantly decreases viral gene expression and viral replication in the heart during myocarditis when female mice are housed in glass cages

We examined whether BPA exposure altered CVB3 levels/ replication in the heart during acute myocarditis when mice were housed in glass cages with glass water bottles. We found that BPA significantly decreased viral gene expression in the heart during acute myocarditis at day 10 pi by qRT-PCR if mice were housed in glass cages (one-way ANOVA, p=0.003). Controlling for multiple comparisons revealed that the 0.5, 5 and 50 μg BPA/kg BW doses significantly decreased viral gene expression at day 10 pi compared to control water (p<0.01, p<0.01, p<0.05, respectively) (**Fig 3A**). We also found that BPA decreased active viral replication by plaque assay in the heart at a 5μg BPA/kg BW dose (p=0.007) (**Fig 3B**). Thus, the decrease in inflammation due to BPA exposure when mice were housed in glass cages could be a result of decreased viral replication in the heart.

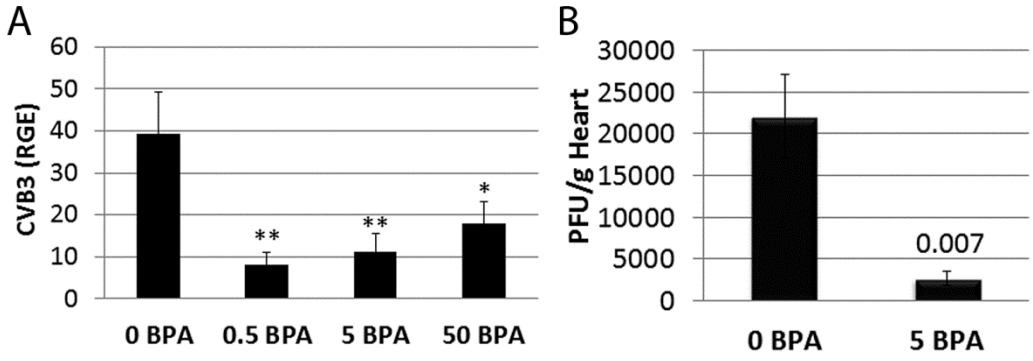


Figure 3. Viral gene expression and replication in the heart were significantly decreased by BPA exposure to female mice housed in glass cages. Female BALB/c mice were given increasing doses of BPA in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0

inflammation examined at day 10 pi, during peak myocarditis. Soy-free bedding and food and glass cages and water bottles were used. BPA exposure was continued from day 0 to harvest at day 10 pi. **A)** CVB3 levels in the heart were measured using qRT-PCR to determine RGE of viral genome compared to the housekeeping gene Hprt. **B)** Viral replication was measured using plaque assay to determine PFU/g heart. Data show the mean +/-SEM, *n*=7-10/group. **A)** Significant differences were assessed using one-way ANOVA for multiple comparisons with post-hoc Student's *t* tests, and **B)** Student's *t* test for viral replication comparing the BPA group to control water. Estimated BPA intakes were determined as follows: 0 (0 BPA), 0.5 (0.5 BPA), 5 (5 BPA), and 50 (50 BPA) μg BPA/kg BW were obtained by dosing water with 0, 2.5, 25 or 250 μg BPA/L, respectively.

BPA exposure does not affect body weight in female mice housed in glass cages

Because BPA exposure has been found to increase the risk for type II diabetes (Bodin 2014, Rezg 2014), we examined whether BPA exposure to mice in glass cages/ water bottles altered the whole body weight of mice. Data are shown for the 5 μg BPA/kg BW dose only. We found that BPA exposure in glass cages/ water bottles had no significant effect on body weight during acute myocarditis at day 10 pi (*p*=0.62) (**Fig 4**).

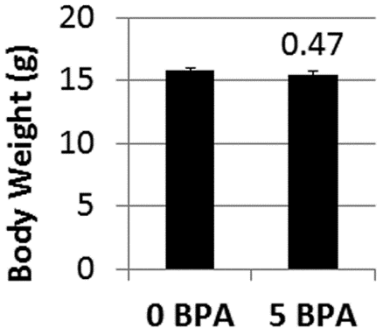


Figure 4. Body weight of female mice was not altered by BPA exposure in glass cages. Female BALB/c mice were exposed to control water (0 BPA) or 5 μg BPA/kg BW (5 BPA) for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and body weight examined at day 10 pi,

during peak myocarditis. Soy-free bedding and food and glass cages and water bottles were used. BPA exposure was continued from day 0 to harvest at day 10 pi. Data are shown as the mean +/-SEM using the Student's *t* test with 38 mice/ group. Estimated BPA intakes were determined as follows: 5 μg BPA/kg BW was obtained by dosing drinking water with 25 μg BPA/L.

BPA exposure in glass cages decreases expression of markers for macrophages, neutrophils, and mast cells in female mice during CVB3 myocarditis

Because BPA exposure to female BALB/c mice in glass cages significantly decreased myocarditis (**Fig 1**), we wanted to examine the specific immune cell types that were altered by BPA exposure. We chose to examine markers for immune cells from the high human relevant dose (i.e., 5 μg BPA/kg BW) in these experiments because it was the same dose that we found a significant increase in myocarditis when plastic cages were used (**Chapter 4, Fig 2**). The effect of BPA exposure on specific cardiac immune cell markers during acute myocarditis at day 10 pi was assessed using qRT-PCR. We found that 5μg BPA/ kg BW exposure to BALB/c female mice housed in glass cages significantly decreased expression of CD45 (all lymphocytes) (p=0.007), CD11b (i.e., macrophages, mast cells, neutrophils, some dendritic cells) (p=7x10^{-5}), GR1 (neutrophils) (p=0.03), F4/80 (macrophages) (p=0.06), CD14 (macrophages and mast cells, part of TLR4 signaling complex) (p=0.03), and cKit/ CD117 (mast cells, stem cells) (p=0.002) in the heart compared to control water without BPA during CVB3 myocarditis (**Fig 5**). The decrease in CD45+ total immune cells and other major populations like GR1+ neutrophils and CD11b+ cells during acute myocarditis following BPA exposure is consistent with the decreased inflammation observed with histology (**Fig 1** and **Fig 2**).

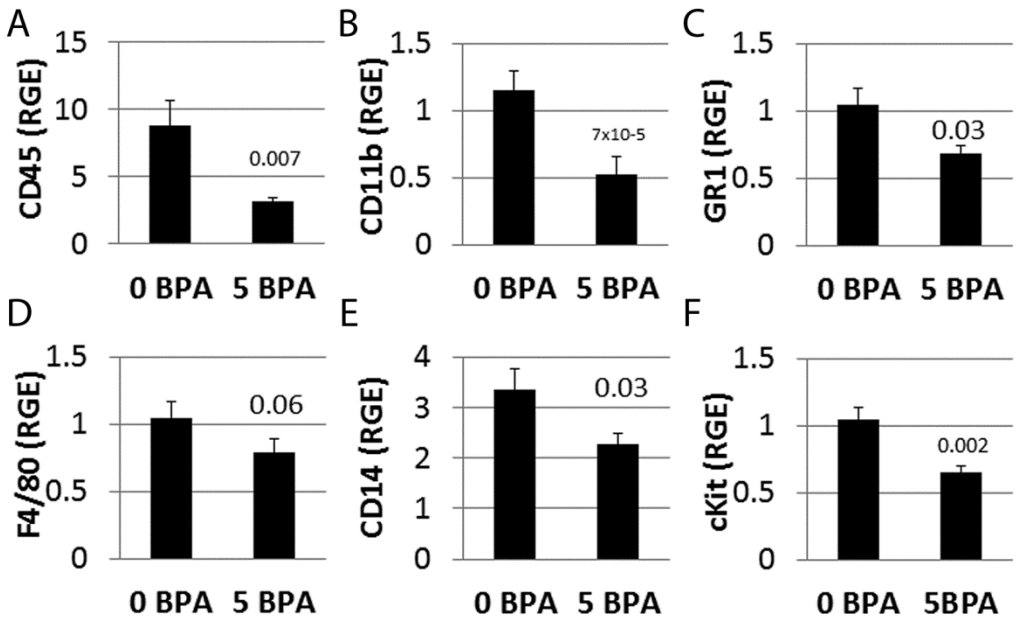


Figure 5. BPA exposure decreases macrophages, neutrophils and mast cells in female BALB/c mice with myocarditis in glass cages. Female BALB/c mice were given 0 or 5 μg BPA (0 BPA vs. 5 BPA) /kg BW in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. Soy-free bedding and food and glass cages and water bottles were used. BPA exposure continued from day 0 to 10 pi. Immune cell populations from the whole heart were examined using qRT-PCR for relative gene expression (RGE) of the genes of interest vs. the housekeeping gene Hprt for **A)** CD45 (total immune cells), **B)** CD11b (includes macrophages, neutrophils, mast cells and some dendritic cells), **C)** GR1 (neutrophils), **D)** F4/80 (macrophages), **E)** CD14 (macrophages and mast cells, part of TLR4 signaling complex), and **F)** cKit/ CD117 (mast cells). Data are shown as the mean +/-SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 8-18 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA exposure in glass cages does not affect T cell marker expression in female BALB/c mice during myocarditis

Because T cells were a dominant immune cell type affected in female BALB/c mice exposed to BPA in plastic cages (**Chapter 4, Fig 7**), we examined whether BPA exposure in glass cages had the same effect. We found that the 5μg BPA/ kg BW exposure to BALB/c female mice housed in glass cages and water bottles had no significant effect on the expression of CD3 (p=0.30), CD4 (p=0.33), CD8 (p=0.15), and Foxp3+ Treg (data not shown, p=0.39) in the heart compared to 0 μg BPA/kg BW (control water) during CVB3 myocarditis by qRT-PCR (**Fig 6**). These data indicate that the immune mechanisms involved in regulating cardiac inflammation in glass vs. plastic cages are different.

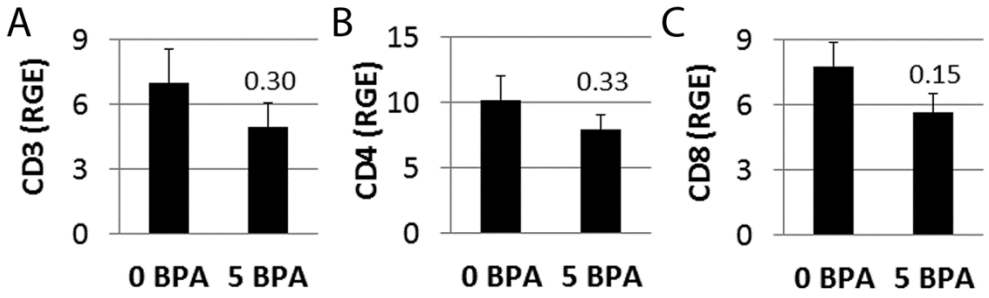


Figure 6. BPA exposure has no effect on T cells in female BALB/c mice with myocarditis in glass cages. Female BALB/c mice were given 0 or 5 μg BPA (0 BPA vs. 5 BPA) /kg BW in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. Soy-free bedding and food and glass cages and water bottles were used. BPA exposure continued from day 0 to 10 pi. Immune cell populations from the whole heart were examined using qRT-PCR for relative gene expression (RGE) of the genes of interest vs. the housekeeping gene Hprt for **A)** CD3+ T cells (total T cells), **B)** CD4+ T cells, and **C)** CD8+ T cells. Data are shown as the mean +/-SEM using a two-tailed Student's t with 9-10 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA exposure in glass cages significantly decreases IFNγ levels, an indicator of a Th1-type immune response, and increases IL-4 an indicator of a Th2-type immune response

In **Chapter 4** we had found that BPA exposure in plastic cages had significantly increased IFNγ and IL-17 levels in the heart during myocarditis (**Chapter 4, Fig 8**). In contrast, we found that BPA exposure in glass cages and water bottles significantly decreased IFNγ (**Fig 7A**) and significantly increased IL-4 levels (**Fig 7B**) in the heart compared to water controls using ELISA. IL-17A was not significantly affected by BPA exposure in glass cages compared to water controls (**Fig 7C**). Typically BALB/c female mice develop a Th2-associated immune response during myocarditis with elevated IL-4 levels (Frisancho-Kiss 2009, Coronado 2012). These results suggest that BPA exposure in glass cages and water bottles further increases a Th2-type immune response during CVB3 myocarditis in female BALB/c mice.

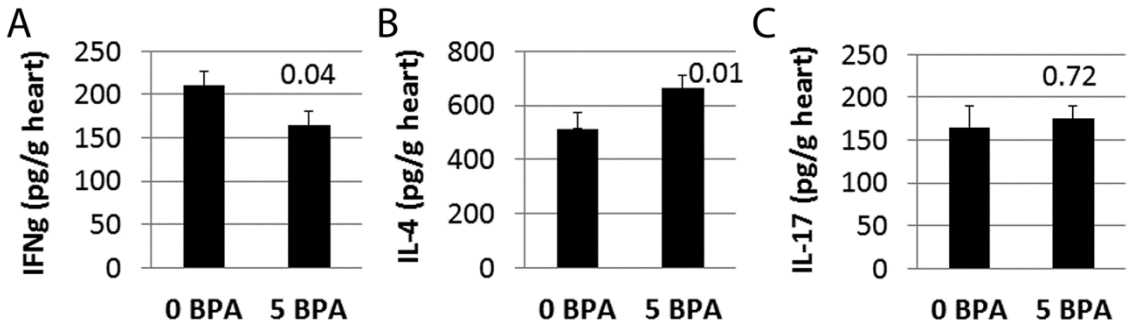


Figure 7. BPA exposure in glass cages decreases cardiac IFNγ levels but increases IL-4 during CVB3 myocarditis in females. Female BALB/c mice were given 0 or 5 μg BPA (0 BPA vs. 5 BPA) /kg BW in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. Soy-free bedding and food and glass cages and water bottles were used. BPA exposure continued from day 0 to 10 pi and homogenized. The supernatant was used to measure **A)** IFNγ, **B)** IL-4, and **C)** IL-17A levels in the heart by ELISA. Data show the mean +/-

SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 14-30 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA exposure in glass cages does not skew the M1 vs. M2 macrophage profile in the heart of females based on arginase-1 and Cxcl9 expression levels

Because we saw a significant change in IFNγ and IL-4 levels in response to BPA in glass cages (**Fig 7**), we wanted to determine whether there was a skewing of M1/M2 macrophages in the heart. Markers of M2 macrophages in mice include arginase-1 (Arg1) while M1 markers include Cxcl9, an inducer of Th1-type immune responses (Siracusa 2008, Fairweather 2009, Abston 2012A). Here we found that female BALB/c mice exposed to 5 μg/kg BW BPA in glass cages had no significant change in the M1 marker Cxcl9 (p=0.51) (**Fig 8A**) or the M2 macrophage marker arginase-1 (p=0.15) (**Fig 8B**) compared to 0 BPA control water by qRT-PCR. These data suggest that the shift in IFNγ and IL-4 in response to BPA in glass cages is not due to a shift in the M1/M2 profile of cardiac macrophages during CVB3 myocarditis.

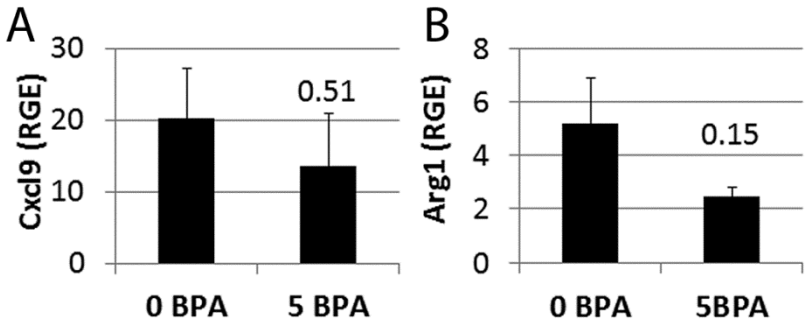


Figure 8. BPA exposure in glass cages does not alter M1 vs. M2 macrophage profile in females during myocarditis. Female BALB/c mice were given 0 or 5 μg BPA (0 BPA vs. 5 BPA) /kg BW in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts

were harvested at day 10 pi. Soy-free bedding and food and glass cages and water bottles were used. BPA exposure continued from day 0 to 10 pi. Relative gene expression (RGE) of **A)** Cxcl9 (an M1 macrophage marker) and **B)** arginase-1 (Arg1) an M2 macrophage markers vs. Hprt controls were assessed in whole hearts using qRT-PCR at day 10 pi comparing 0 and 5 μg BPA/kg BW groups. Data show the mean ±SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 9-10 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA exposure in glass cages decreases expression of the mast cell anaphylatoxin receptor C3aR1 and C4b levels in the heart of females

We found that BPA exposure in glass cages significantly decreased the expression of markers for macrophages and mast cells as well as CD11b/ CR3 during myocarditis (**Fig 5**), a receptor that is highly expressed on macrophages and mast cells during myocarditis (Fairweather 2006, Frisancho-Kiss 2009, Onyimba 2011, Coronado 2012, Fairweather 2014). For this reason we examined the level of other complement components using qRT-PCR. We found that BPA exposure in glass cages significantly decreased the expression of C4b (p=0.04) (**Fig 9A**) and the mast cell anaphylatoxin receptor C3aR1 (p=0.02) (**Fig 9B**) in the heart during myocarditis compared to the water control, but did not significantly alter C5aR1 (p=0.67) (**Fig 9C**). Downregulation of C3aR1 suggests a decrease in mast cell activation.

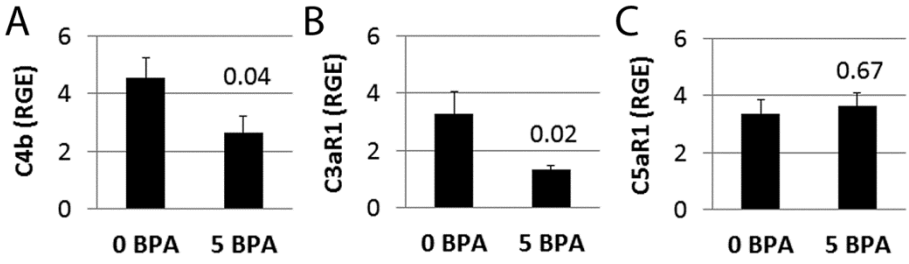
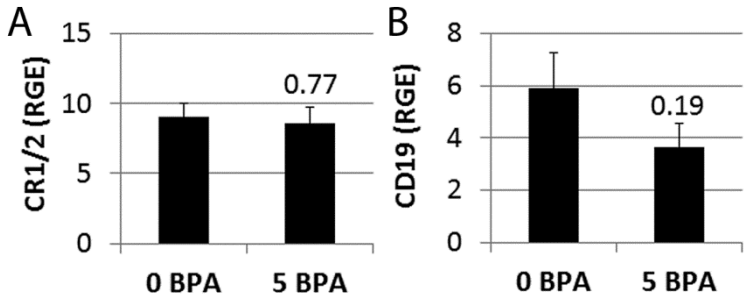


Figure 9. BPA exposure in glass cages decreases C4b and mast cell activation via C3aR1 in females. Female BALB/c mice were given 0 or 5 μg BPA (0 BPA vs. 5 BPA) /kg BW in their

drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. Soy-free bedding and food and glass cages and water bottles were used. BPA exposure continued from day 0 to 10 pi. Relative gene expression (RGE) of complement genes vs. the housekeeping control Hprt was examined for **A)** C4b **B)** C3aR1, and **C)** C5aR1 mRNA by qRT-PCR at day 10 pi comparing 0 to 5 μg BPA/kg BW estimated intake groups. Data show the mean +/-SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 9-18 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA exposure has no effect on complement regulator CR1/2 or CD19 in females

CR1 is the primary receptor in the heart that regulates complement activation and protects against autoimmune and viral myocarditis in mice (Kaya 2001, Fairweather 2006). CR1 deficient mice develop severe inflammation and fibrosis and rapidly progress to DCM and heart failure following CVB3 infection, indicating the critical role for CR1 in dampening the immune response to CVB3 (Fairweather 2006). For this reason we examined CR1 expression in the heart during myocarditis following BPA exposure in glass cages using qRT-PCR. CR2 forms a B cell receptor complex with CD19 and CD81 needed for complement activation of B cells. So we also examined the level of CD19, a marker often used to detect B cells using qRT-PCR. We found that neither CR1/2 (p=0.77) (**Fig 10A**) nor CD19 (p=0.19) (**Fig 10B**) expression was significantly altered during myocarditis by BPA exposure compared to water controls in mice housed in glass cages, indicating that BPA exposure in glass cages does not alter complement regulation.



F**igure 10. BPA exposure in glass cages has no effect on the complement regulation via CR1/CR2/CD19 in females.** Female BALB/c mice were given 0 or 5 μg BPA (0 BPA vs. 5 BPA) /kg BW in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. Soy-free bedding and food and glass cages and water bottles were used. BPA exposure continued from day 0 to 10 pi. Relative gene expression (RGE) of **A)** CR1/2 and **B)** CD19 (part of the CR2 signaling complex on B cells) were compared to Hprt controls in the heart by qRT-PCR at day 10 pi comparing 0 to 5 μg BPA/kg BW groups. Data show the mean +/-SEM using a two-tailed Student's *t* test with 9-10 mice/ group. Estimated BPA intakes were determined as follows: 0 μg BPA/kg BW obtained by dosing water with 0 μg BPA/L water and 5 μg BPA/kg BW obtained by dosing water with 25 μg BPA/L water.

BPA exposure in glass cages decreases TLR4/ IL1R pathway activation in the heart of females during myocarditis

We showed previously that components of IL-1R signaling (i.e., TLR4, IL-1R antagonist, caspase-1, IL-1β and IL-18) are upregulated in the heart during CVB3 myocarditis in male mice and that testosterone amplifies this response on mast cells and macrophages during the innate and adaptive immune response (Coronado 2012, Fairweather 2015, Frisancho 2006, Frisancho 2007, Frisancho 2009, Roberts 2013). Because BPA exposure in glass cages decreased the expression of markers of macrophage and mast cell

populations in the heart during myocarditis (**Fig 5**), we examined its effect on TLR4 signaling components. We found that BPA exposure in glass cages significantly decreased expression of TLR4 (p=0.03), caspase-1 (p=0.0007), and IL-1β (p=0.01) (**Fig 11A-C**). ST2 receptor expression, which is a member of the TLR/IL-1R signaling family that includes IL-1R2 and is expressed on mast cells and macrophages, was also significantly decreased in the heart during myocarditis following BPA exposure in glass cages when measured using qRT-PCR; IL-1R2 (p=0.02) (**Fig 11D**), ST2 (p=0.002) (**Fig 11E**). Additionally, sST2 was significantly decreased in the heart by ELISA (p=0.005) (**Fig 11F**). These data indicate that BPA strongly influences TLR4/IL1R family members.

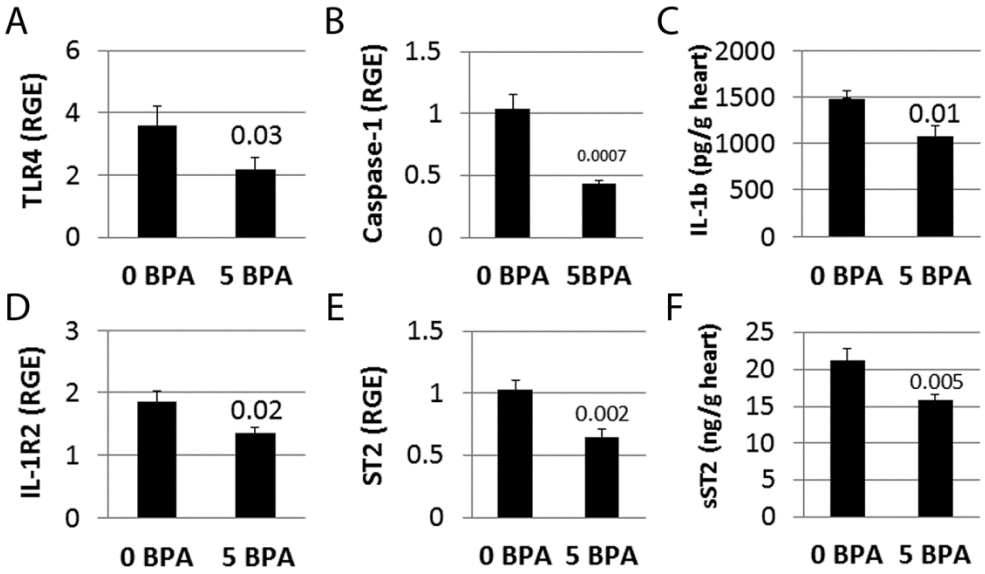


Figure 11. BPA exposure in glass cages inhibits the inflammasome in females during myocarditis. Female BALB/c mice were given 0 or 5 μg BPA (0 BPA vs. 5 BPA) /kg BW in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. Soy-free bedding and food and glass cages and water bottles were used. BPA exposure continued from day 0 to 10 pi. Relative gene expression (RGE) of inflammasome genes vs. the housekeeping control Hprt for **A)** TLR4 (n=7-10/group), **B)** caspase-1 (n=8-10/group), **D)** IL-1R2

(*n*=7-10/group), and **E)** ST2 receptor (*n*=5-10/group) were examined in whole hearts by qRT-PCR at day 10 pi comparing 0 to 5 μg BPA/kg BW groups. **C)** IL-1β (*n*=14-20/group) and **F)** sST2 (*n*=24-30) levels were determined using ELISA from homogenized heart supernatants. Data show the mean +/-SEM using a two-tailed Student's *t* or Mann-Whitney rank test. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA exposure in glass cages decreases factors associated with cardiac remodeling during myocarditis in females

Because BPA exposure in glass cages was found to decrease IL-1β and markers for mast cells, macrophages, and complement activation, we examined whether BPA altered factors associated with cardiac remodeling during acute myocarditis- a timepoint when profibrotic genes/ proteins are elevated in male BALB/c mice with myocarditis but histologic fibrosis (i.e., collagen deposition) is not yet present (Coronado 2012). We found that BPA exposure in glass cages did not significantly alter expression of Timp1 (**Fig 12A**) in the heart, but did significantly decrease Serpin A3n (**Fig 12B**) and TGFβ1 (**Fig 12C**) levels. BPA exposure in glass cages also significantly decreased the profibrotic cytokine IL-1β in the heart by ELISA (**Fig 11C**). These data suggest that BPA exposure in glass cages reduces remodeling gene expression in the heart during acute myocarditis.

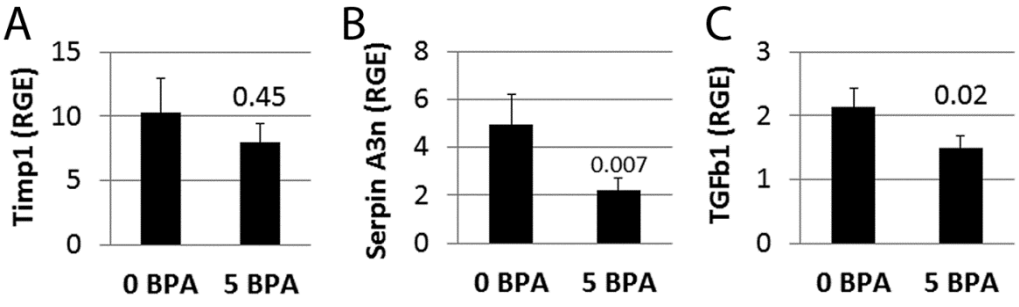


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Figure 12. BPA exposure in glass cages decreases remodeling factors in the heart during myocarditis in females. Female BALB/c mice were given 0 or 5 μg BPA (0 BPA vs. 5 BPA) /kg BW in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. Soy-free bedding and food and glass cages and water bottles were used. BPA exposure continued from day 0 to 10 pi. Relative gene expression (RGE) of **A)** Timp1, **B)** Serpin A3n and **C)** TGFβ1 vs. the housekeeping gene Hprt were analyzed in whole hearts by qRT-PCR at day 10 pi comparing 0 to 5 μg BPA/kg BW groups. Data show the mean +/-SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 9-10 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA in glass cages decreases ERα expression in the heart during myocarditis in females

We showed in **Chapter 4** that BPA exposure in plastic cages activated ERα and ERβ in Western blots (**Chapter 4, Fig 24 and Fig 25**). By qRT-PCR, BPA was found to increase ERα and decrease ERβ expression in the heart in plastic cages (**Chapter 4, Fig 23**). So in this Chapter we wanted to examine the effect of BPA exposure when glass cages and water bottles were used. We found that ERα expression was significantly decreased in the heart of females after BPA exposure in glass cages by qRT-PCR (p=0.04) (**Fig 13A**), but ERβ, ERRγ and AR expression were not significantly altered (**Fig 13**). These data indicate that BPA exposure in glass cages decreases ERα in female BALB/c mice, similar to what we found using plastic cages (**Chapter 4, Fig 23A**). However, the relationship of ERα signaling to cardiac inflammation is less clear because BPA exposure at this dose in plastic cages increased myocarditis while the same dose in glass cages decreased myocarditis. Perhaps the ratio of ERα to ERβ expression is important in determining the effect of ERs on cardiac

inflammation, with BPA exposure in plastic cages in females decreasing ERα but increasing ERβ while BPA exposure in glass cages only decreased ERα. Future studies will need to examine ER activation using Western blot in female mice exposed to BPA in glass cages.

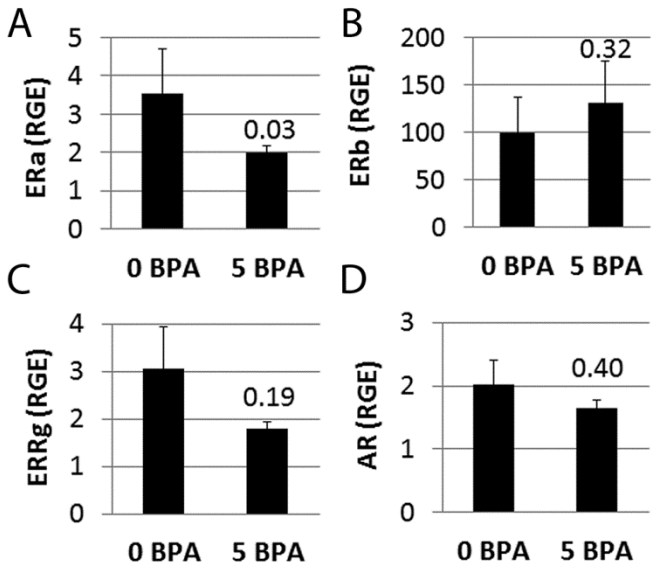

Figure 13. BPA exposure in glass cages decreases ERα expression in the heart during myocarditis in females. Female BALB/c mice were given 0 or 5 μg BPA (0 BPA vs. 5 BPA) /kg BW in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. Soy-free bedding and food and glass cages and water bottles were used. BPA exposure continued from day 0 to 10 pi. Relative gene expression (RGE) of **A)** ERα, **B)** ERβ, **C)** ERRγ and **D)** AR vs. the housekeeping gene Hprt were analyzed in whole hearts by qRT-PCR at day 10 pi comparing 0 to 5 μg BPA/kg BW groups. Data show the mean +/-SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 9-10 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA exposure does not alter VDR or enzymes that metabolize VitD in females

We previously showed that VDR signaling is protective during acute myocarditis in females and increases myocarditis in males (**Chapter 3, Fig 3**). Additionally, we found that BPA exposure in plastic cages significantly decreased VDR expression in the heart of female BALB/c mice with myocarditis (**Chapter 4, Fig 27**). BPA exposure in glass cages, however, did not significantly alter VDR expression or alter the enzymes involved in vitamin D metabolism in the heart of female BALB/c mice (**Fig 14**).

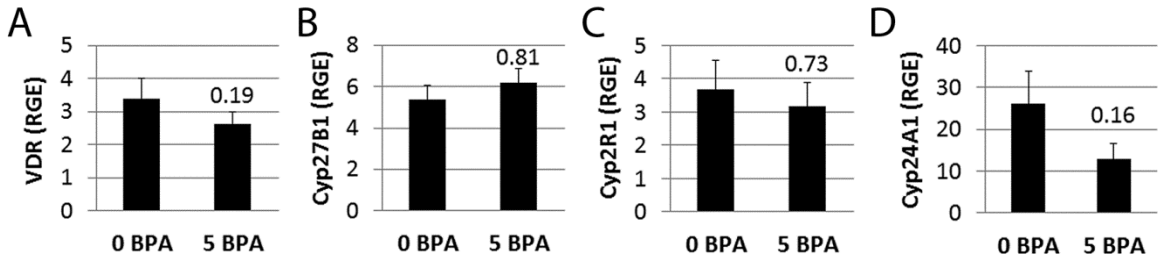

Figure 14. BPA exposure in glass cages does not alter VDR expression in the heart of female mice with myocarditis. Female BALB/c mice were given 0 or 5 μg BPA (0 BPA vs. 5 BPA) /kg BW in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. Soy-free bedding and food and glass cages and water bottles were used. BPA exposure continued from day 0 to 10 pi. Relative gene expression (RGE) of **A)** VDR, **B)** Cyp27B1, **C)** Cyp2R1 and **D)** Cyp24A1 vs. the housekeeping gene Hprt were analyzed in whole hearts by qRT-PCR at day 10 pi comparing 0 to 5 μg BPA/kg BW groups. Data show the mean +/- SEM using a two-tailed Mann-Whitney rank test with 5-10 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

Free BPA and BPA-glucuronide levels in the urine of females were not significantly altered between 0 and 5 μg BPA/kg BW dose

To determine BPA levels released in the urine of female BALB/c mice exposed to BPA in glass cages we examined free BPA and BPA-glucuronide levels in urine by LC-

MS/MS with collaborators at the University of Kentucky. We did not find a significant difference in urine BPA levels between BPA exposed female mice compared to control water (**Fig 15**). One potential cause for this result could be that the urine was stored for a year in plastic tubes, which may have leached plastics/ BPA/ chemicals into the urine confounding the results. Future studies analyzing BPA levels in water, urine, and blood will be collected in glass containers in order to prevent potential BPA leaching during storage that could affect the analysis.

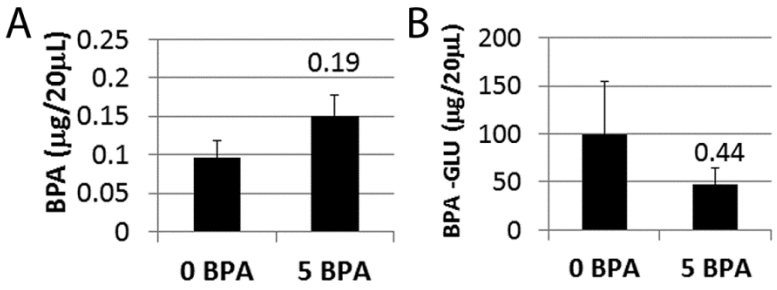

Figure 15. BPA concentration in urine of females detected by LC-MS/MS was not altered by BPA dose. A) Free BPA and **B**) BPA-glucuronide (BPA-GLU) levels were assessed in urine of female BALB/c mice treated with control water or BPA treated water. Urine was collected from individual mice on day 0, 3, 7, and 10 pi and days into plastic tubes, pooled for each mouse, and stored at -80C. Samples were sent to collaborators at University of Kentucky for analysis using LC-MS/MS. Significance was calculated using Student's *t* test or Mann-Whitney rank test comparing BPA exposure to 0 control group. Data show the mean ±SEM using pooled samples from 3 mice/ group. 5 µg BPA/kg BW was obtained by dosing water with 25 µg BPA/L.

BPA exposure in glass cages did not alter hormone receptor levels in the heart of uninfected female BALB/c mice

We wanted to determine if BPA exposure without virus could influence the gene expression of hormone receptors in the heart. Female 6-8 week old BALB/c mice were exposed to 5 μg BPA/kg BW for 2 weeks plus 10 days, so that mice were age-matched to previous experiments in this Chapter. They also received soy-free bedding and food. We found that BPA exposure had no significant effect on sex hormone expression levels in the heart compared to control water when myocardial inflammation was absent (**Fig 16**). We also investigated if BPA could influence the VDR and Cyps without infection and saw no change in expression (data not shown). These data suggest that changes in sex hormone receptor expression that we observed due to BPA exposure during CVB3 myocarditis are present on/in cardiac immune cells rather than cardiac myocytes, fibroblasts, and/or endothelial cells. It is also worth noting that BPA exposure alone does not cause myocarditis. Thus, BPA exposure prior to CVB3 infection (co-exposure) is able to significantly alter myocardial inflammation.

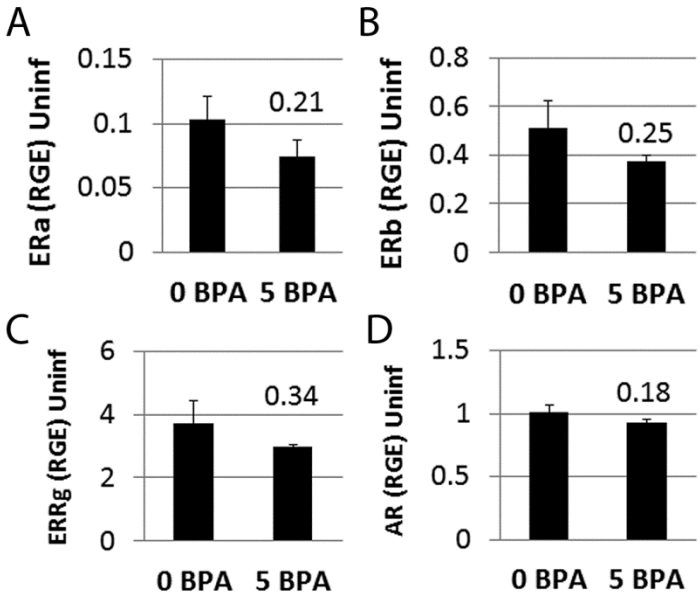

Figure 16. BPA exposure in glass cages had no influence on hormone receptor expression in the heart of uninfected female BALB/c mice. Female BALB/c mice were given 0 or 5 μg BPA (0 BPA

vs. 5 BPA) /kg BW in their drinking water for 2 weeks plus 10 days and then hearts were harvested. Soy-free bedding and food and glass cages and water bottles were used. Relative gene expression (RGE) of **A)** ERα, **B)** ERβ, **C)** ERRγ and **D)** AR vs. the housekeeping gene Hprt were analyzed in whole hearts by qRT-PCR comparing 0 to 5 μg BPA/kg BW groups. Data show the mean +/-SEM using a two-tailed Student's *t* with 9-10 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

Discussion

In this study we found that BPA exposure to adult female mice housed in glass cages significantly decreased myocarditis at day 10 pi compared to control water for all doses of BPA (**Fig 1**)**.** This indicates that there was no dose effect for BPA exposure on myocarditis when females were housed in glass cages at the doses investigated. In the future we will examine lower doses of BPA to determine if there is truly no dose response. Additionally, BPA was able to significantly decrease myocarditis at the lowest dose (human relevant dose) that we tested in drinking water, 2.5 μg BPA/L which is equivalent to an estimated intake of 0.5 μg BPA/kg BW. Similar to our findings that BPA exposure decreased markers of CD11b and F4/80 macrophages (**Fig 5**), BPA exposure was found to decrease macrophages in the pancreas in an animal model of type I diabetes using BPA-free water bottles (Bodin 2013). Note that plastic BPA-free water bottles will contain BPS and potentially other plastics that can leach into the water (see **Chapter 9**). Thus, BPA exposure in glass cages appears to have "increased" the typical cardioprotective female immune response associated with decreased myocarditis in females. This unexpected finding could be due to the glass caging possibly decreasing the stress levels of the mice due to being able to better see out of the caging, having increased room, and/or being able to pull food out of the ceramic bowl. In the future, we could further investigate the effect of the glass caging on the stress levels in mice by examining their circulating glucorticoid levels.

Decreased myocarditis following BPA exposure in female BALB/c mice in glass cages could also be due to the reduction in viral replication that we observed in the heart (**Fig 3**). Additionally, BPA has been found to have an influence on body weight in animal models,

both increasing and decreasing weight depending on the dose (Markey 2001), but we found that BPA exposure during acute myocarditis did not affect the body weight of BALB/c mice.

BPA exposure significantly decreased ERα levels in the heart of females during myocarditis, but AR, ERβ and ERRγ levels were unchanged according to qRT-PCR (**Fig. 13**). This was an unexpected finding because we hypothesized that ERα signaling should be protective. Inflammatory signaling pathways known to be down-regulated during myocarditis and DCM in females and that are associated with mast cell activation and increased inflammation in males (i.e., TLR4, complement/anaphylaxis, Serpin A3n) (Kaya 2001, Fairweather 2006, Coronado 2012) were significantly decreased in females after BPA exposure in this study. We also found that BPA exposure increased the Th2 cytokine, IL-4, and decreased the Th1 cytokine, IFNγ (**Fig 7**), which was also found by Yan et al. They found that BPA exposure increased Th2-type cytokine release (i.e., IL-4, IL-10, and IL-13) in response to a bacterial infection (Yan 2008). Additionally, Teixeira et al found that BPA treatment of human peripheral blood monocyte-derived macrophages *in vitro* induced an M2 macrophage phenotype (Teixeira 2015). We did not observe a change in M1 vs. M2 markers in this study (**Fig 8**), but we only examined one marker for each cell type and so more M1 vs. M2 markers should be examined in the future.

To determine if co-exposure with BPA plus CVB3 infection to induce myocarditis was necessary for altering ER expression in the heart, we dosed BALB/c female mice with BPA without virus (in glass cages and water bottles with soy-free food and water for the same amount of time as other experiments) and found that BPA exposure did not significantly alter cardiac expression of ERs or the AR unless myocarditis was present. Thus, co-exposure is necessary. Perhaps because the sex hormone receptor changes that we are

observing are on/in infiltrating immune cells rather than resident cardiac cells like cardiomyocytes. Other investigators have made similar observations. Yurino et al found that BPA exposure alone had no significant effect on lymphocyte cell numbers or percentages in the spleen or peritoneal cavity (Yurino 2004).

In this study we examined urinary BPA levels from pooled samples over time during acute myocarditis and then tested the urine for unconjugated and metabolize BPA by LC-MS/MS. We found that the average unconjugated BPA concentration in BALB/c females housed in glass cages was 0.0014 μg/L. This value is low. Also there was no significant difference between the 0 control urine and unconjugated BPA exposed urine (there was also no difference in glucuronidated BPA). This indicates that something is wrong with our collection and/or method of detecting BPA in the urine. Glucuronidation is known to be the primary method of BPA metabolism (Corrales 2015). But BPA can also be metabolized by sulfation. So, one possibility to explain our findings is that BPA sulfation may be a more important method of metabolism of BPA than glucuronidation during acute CVB3 myocardits and so our method is not detecting the metabolized levels accurately. In future studies this issue will need to be examined. Additionally, in the future we plan to examine BPA levels in the serum. Unconjugated BPA levels in the urine of people with "normal" BPA exposure have been found to be 2.16 μg/L compared to 5.45 μg/L in individuals who are exposed to higher levels of BPA through thermal receipts as an occupational exposure (Hehn 2016). Because populations are likely to be exposed to BPA and other plastics from a wide variety of sources (i.e., food, water, receipts, photocopy paper, etc.) and exposure routes (i.e., oral, cutaneous, inhalation) future experiments will need to examine the effect of BPA exposure from multiple sources on inflammatory diseases like myocarditis. Although BPA

exposure to female mice appears to protect against CVB3 myocarditis, it is likely that most of the population is exposed to multiple forms of endocrine disruptors. Regardless of the type of cage, BPA exposure does appear to strongly alter myocardial inflammation in female BALB/c mice.

Chapter 7

BPA increases myocarditis in male BALB/c mice housed in glass cages

Abstract

Myocarditis is an inflammatory heart disease that leads to DCM and heart failure. Sex hormones play a vital role in the development of myocarditis with testosterone driving disease in males. Since myocarditis is influenced by sex hormones, it is highly probable that endocrine disruptors, which interfere with natural hormones, will play a part in the progression of the disease. The human population is exposed to the endocrine disruptor BPA from plastics, such as water bottles, plastic food containers, and receipts, for example. BPA is thought to also be mildly androgenic and could elicit effects through both ERs and the AR. To our knowledge no one has examined the role of endocrine disruptors like BPA on myocarditis in male mice. In this study we found that all doses of BPA increased myocarditis in male BALB/c mice. Using the EPA reference dose, we found that BPA exposure significantly increased VDR and decreased ERRγ expression in the heart. We found that expression of markers for macrophages, neutrophils, and T cells were significantly increased by BPA exposure while cKit (a marker for mast cells) was decreased. The increase in myocarditis was associated with an increase in the TLR4 signaling pathway, IL-1R2, complement, C3aR1, C5aR1, and Timp-1. Thus, BPA exposure in male BALB/c mice upregulated all the key immune processes that are known increase myocarditis and lead to DCM in males.

Introduction

BPA is primarily thought to mediate its effects through ERs, but it has also been shown that it can bind the AR as an agonist at a very low level (at about 20% of the binding efficiency of testosterone) (Molina-Molina 2013). However, BPA has also been found to act as an antagonist for the AR (Molina-Molina 2013). Interestingly, urinary BPA levels from NHANES data from 2003-2004 found that men had significantly higher BPA levels than women (Lakind 2008), indicating that BPA doses may be higher in men or metabolized differently than women. It has been shown that UDP-glucuronsyltransferases and sulfotransferances differ by tissue and sex in mice (Alnouti 2006, Buckley 2007).

Although AR expression is higher in men than women, and women express higher levels of ERs than men, cells express both ERs and the AR and it is likely the ratio of these receptors that determines cell function. Interestingly, it has been shown that ERβ is more highly expressed than ERα in the male reproductive system (Weihua 2001, Acconcia 2015). BPA has also been found to decrease ERα expression in the spleens of male rats (Miao 2008). Similarly, BPA treatment of male human T cell lymphoblast lines in culture was found to increase ERβ expression using qRT-PCR (Cipelli 2014).

Few studies have examined whether BPA exposure could have an effect on normal physiology or disease in males. Some recent studies have found that BPA exposure is associated with male infertility and a low sperm count (Vitku 2015). BPA has also been found to significantly increase the number of microglia (which are CD11b+ brain macrophages) in the brain of male rats, but not in females (Rebuli 2016). Because men are exposed to BPA in the environment, BPA can influence the AR, and ERs may also affect

myocarditis in males, we decided to examine the effect of BPA exposure on male BALB/c mice using glass cages and water bottles and soy-free food and bedding.

Results

BPA exposure increases CVB3 myocarditis in male BALB/c mice in glass cages at all doses

Many studies have found that BPA increases cardiac, autoimmune and inflammatory diseases (Weinstock-Guttman 2003, Orton 2004, Kendziorski 2012, Melzer 2012, Shankar 2012, Gao 2014, O'Brien 2014, Jochmanova 2015, Ranciere 2015, Han 2016, He 2016). However, few studies have examined the effect of BPA exposure on males (Kendziorski 2012, Vitku 2015, Rebuli 2016). It is well established that sex hormones influence cardiac diseases such as myocarditis (Coronado 2012). However, no studies have assessed whether BPA exposure could alter myocarditis in male mice. To assess the effect of BPA exposure on CVB3 myocarditis in male mice, 6-8 week old male BALB/c mice were given the same doses of BPA in water for 2 weeks using glass cages and water bottles prior to infection with CVB3 on day 0 as were given to female BALB/c mice in **Chapter 4 and 5**. Soy-free food and bedding were used. BPA exposure was continued from day 0 to 10 pi. Cardiac inflammation was assessed histologically at day 10 pi during acute myocarditis by determining % inflammation by H&E staining of heart sections.

We found that the exposures of 2.5, 25 and 250 μg BPA/L water, which is equivalent to an estimated intake of 0.5, 5 and 50 μg BPA/kg BW, respectively, significantly increased myocarditis at day 10 pi compared to control water containing no BPA in male BALB/c mice housed in glass caging ($p<0.0007$) (**Fig 1**). Controlling for multiple comparisons found a significant increase in myocarditis with BPA exposure compared to water control for 0.5 μg BPA/kg BW ($p<0.01$), 5 μg BPA/kg BW ($p<0.001$), and 50 μg BPA/kg BW ($p<0.05$). Post-hoc analyses using Student's *t* tests indicated that all BPA doses significantly increased

myocarditis compared to control water; human relevant dose of 0.5 μg BPA/kg BW (p=0.001), high human relevant dose of 5 μg BPA/kg BW (p=0.0003), and the EPA reference dose of 50 μg BPA/kg BW (p=0.005) (**Fig 1**). Representative examples of the inflammation in males are shown in **Figure 2**.

In all further analyses shown in this Chapter the *EPA reference dose* was used. This is because when the histology was originally assessed it was thought that this dose had the largest/ most significant effect. Thus, when the experiment was repeated to obtain tissue to conduct qRT-PCR, the 50 μg BPA/kg BW dose was used. When histology experiments were repeated it was realized that a mistake had occurred and that actually the 5 μg BPA/kg BW dose was most significantly increased, similar to results found for female BALB/c mice housed in plastic cages in **Chapter 4**. In future experiments in male mice with glass cages we will analyze the immune mechanisms involved with the 5 μg BPA/kg BW dose.

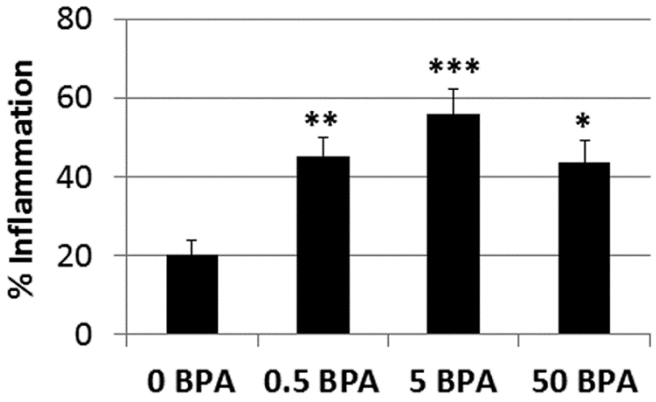

Figure 1. BPA exposure increases myocarditis in BALB/c male mice housed in glass cages. Male BALB/c mice were given 0 (0 BPA), 0.5 (0.5 BPA), 5 (5 BPA), and 50 (50 BPA) μg BPA/kg BW in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and cardiac inflammation examined at day 10 pi, during peak myocarditis. Soy-free bedding and food and glass cages and water bottles were used. BPA exposure continued from day 0 to 10 pi. Myocarditis was

assessed as the % inflammation in the heart compared to the overall size of the heart section by histology using an eyepiece grid. Estimated BPA intakes were determined as follows: 0, 0.5, 5, and 50 μg BPA/kg BW were obtained by dosing water with 0, 2.5, 25 or 250 μg BPA/L, respectively. One-way ANOVA found a significant difference existed between groups (p<0.0007). After controlling for multiple comparisons, the 0.5 (**, p<0.01), 5 (***, p<0.001), and 50 (*, p<0.05) μg BPA/kg BW dose were significantly increased compared to control water. Data show the mean +/- SEM of 10 mice/ group. The same finding was observed when the experiment was repeated for a total of 18-20 mice/ group.

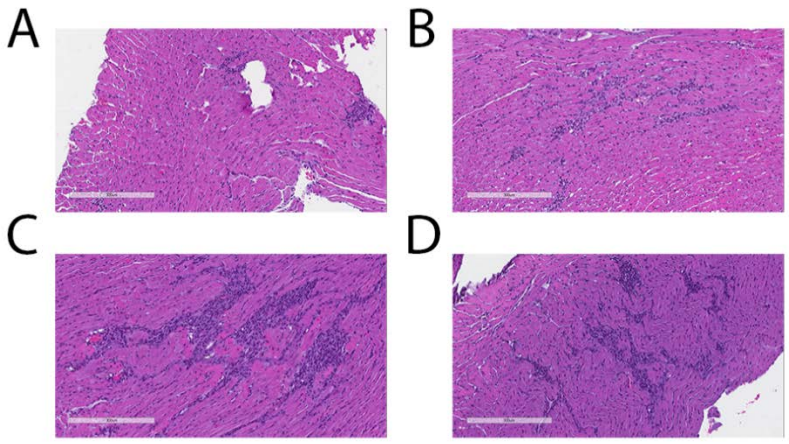

Figure 2. Representative photos of the effect of BPA exposure on CVB3 myocarditis in male mice in glass cages. Male BALB/c mice were given increasing doses of BPA in drinking water using glass cages and water bottles for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and cardiac inflammation examined at day 10 pi, during peak myocarditis. Soy-free bedding and food were used. BPA exposure continued from day 0 to 10 pi. Inflammation in the heart was assessed using H&E stain where cardiac tissue appears pink and inflammation dark purple. Representative photos depict **A)** 0, **B)** 0.5, **C)** 5, and **D)** 50 μg BPA/kg BW. The grey bar is 300μm in length.

BPA exposure does not significantly alter viral gene expression in the heart during myocarditis in males

We examined whether BPA exposure altered CVB3 gene expression in the heart during acute myocarditis in male mice in glass cages. We used a primer/probe set directed to the CVB3 genome according to Antoniak et al. (Antoniak 2013). We found that BPA exposure had no significant effect on viral gene expression levels in the heart during acute myocarditis in males at day 10 pi by qRT-PCR (p=0.74) (**Fig 3**). Thus, increased myocardial inflammation from BPA exposure in males using glass cages was not due to increased viral replication during acute CVB3 myocarditis.

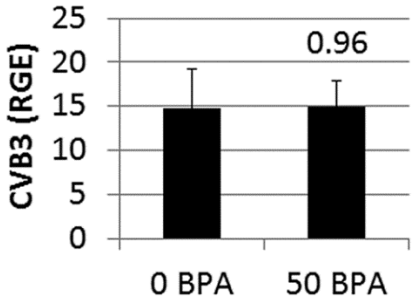

Figure 3. Viral gene expression in the heart was not altered by BPA treatment of male mice in glass cages. Male BALB/c mice were given 0 (0 BPA) or 50 (50 BPA) μg BPA/kg BW in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. Soy-free bedding and food and glass cages and water bottles were used. BPA exposure continued from day 0 to 10 pi. CVB3 levels in the heart were measured using qRT-PCR to determine relative gene expression (RGE) of viral genome compared to the housekeeping gene Hprt. Data show the mean +/-SEM, n=10/group Significant differences between groups were assessed using Student's t test. 50 μg BPA/kg BW was obtained by dosing water with 250 μg BPA/L.

BPA exposure increases markers of individual immune cell types in males in glass cages during myocarditis

In order to determine the effect of BPA exposure on individual immune cell types in the heart during CVB3 myocarditis in male mice housed in glass cages we conducted qRT-PCR of whole hearts (i.e., not isolated cells) at day 10 pi using mice exposed to the EPA reference dose. We found that the 50 µg BPA/kg BW exposure dose significantly increased expression of CD45 (p=0.01), CD11b (p=0.03), F4/80 (p=0.02), GR1 (p=0.04), CD14 (p=0.03), CD3 (p=0.006), CD4 (p=0.01), CD8 (p=0.03), and Foxp3 (data not shown, p=0.0002) in the heart compared to 0 BPA control water during CVB3 myocarditis (**Fig 4**). However, this dose of BPA had no significant effect on cKit/ CD117 (i.e., mast cells) (p=0.08) expression in the heart compared to 0 BPA control water during CVB3 myocarditis (**Fig 4F**). This increase in immune cell populations determined by qRT-PCR following BPA exposure is consistent with the increased inflammation we observed with histology (**Fig 1**).

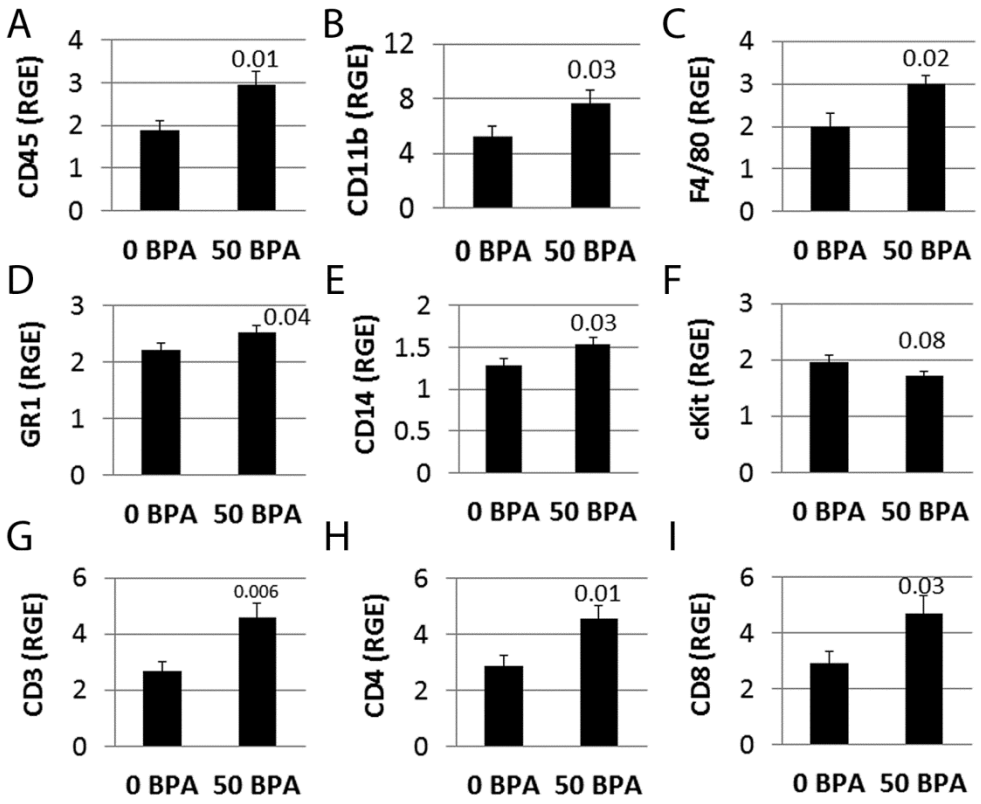

Figure 4. Immune cell markers were significantly increased by BPA exposure in males in glass cages. Male BALB/c mice were given 0 (0 BPA) or 50 (50 BPA) μg BPA/kg BW in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. Soy-free bedding and food and glass cages and water bottles were used. BPA exposure continued from day 0 to 10 pi. Immune cell populations in the heart were examined using qRT-PCR for relative gene expression (RGE) of the gene of interest vs. the housekeeping gene Hprt for CD45 (total immune cells), CD11b (includes macrophages, neutrophils, mast cells and some dendritic cells), F4/80 (macrophages), GR1 (neutrophils), CD14 (part of TLR4 signaling complex on macrophages and mast cells), CD3 (all T cells), CD4 (CD4+ T cells), and CD8 (CD8+ T cell). Data are shown as the mean +/-SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 10 mice/ group. 50 μg BPA/kg BW was obtained by dosing water with 250 μg BPA/L.

BPA exposure does not alter M1 vs. M2 macrophage markers in the heart of males in glass cages during myocarditis

Markers of M2 macrophages in mice include arginase-1 (Arg1) and Ym1 (eosinophilic protein from chitinase family), while M1 markers include Cxcl9 and Cxcl10 (Siracusa 2008, Fairweather 2009). Here we found that male BALB/c mice exposed to the EPA reference dose of BPA had no significant change in M2 macrophage markers arginase-1 (p=0.59) and Ym1 (p=0.22) or M1 macrophage markers Cxcl9 (p=0.13) and Cxcl10 (p=0.19) compared to 0 BPA control water by qRT-PCR (**Fig 5**).

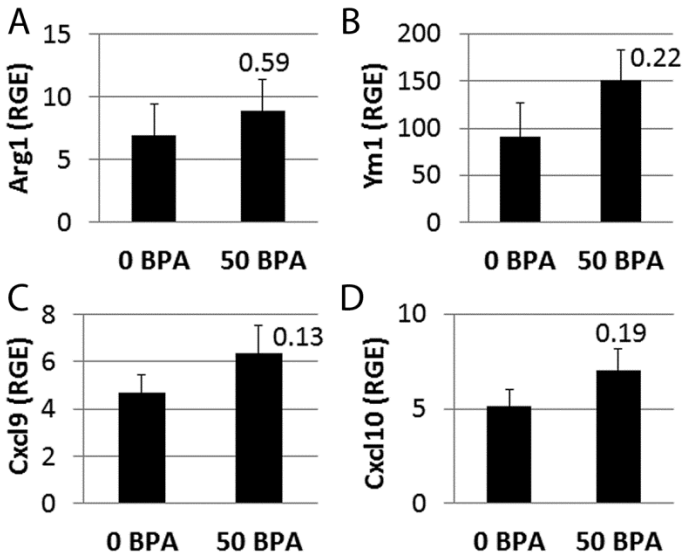

Figure 5. BPA exposure does not change the relative gene expression of M1 or M2 macrophage markers in males in glass cages during myocarditis. Male BALB/c mice were given 0 (0 BPA) or 50 (50 BPA) μg BPA/kg BW in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. Soy-free bedding and food and glass cages and water bottles were used. BPA exposure continued from day 0 to 10 pi. Relative gene expression (RGE) of **A)** M2 macrophage markers (Arg1, Ym1) and **B)** M1 markers (Cxcl9, Cxcl10) vs. Hprt controls were assessed in whole hearts using qRT-PCR at day 10 pi comparing 0 and 50 μg BPA/kg BW groups. Data show the mean ±SEM using a two-tailed Student's *t* test with 10 mice/ group. 50 μg BPA/kg BW was obtained by dosing water with 250 μg BPA/L.

BPA exposure increases expression of mast cell anaphylatoxin receptors C3aR1 and C5aR1 in males

Because of the important role of the complement pathway in the pathogenesis of CVB3 myocarditis in male mice (Fairweather 2006) and because the EPA reference dose increased CD11b/CR3 expression in males with myocarditis (**Fig 4B**), we examined the expression of complement components and receptors in the heart of BPA exposed male mice

with myocarditis that had been housed in glass cages. We found that BPA exposure had no significant effect on C3 (p=0.29) or C4b (p=0.13), but significantly increased expression of the mast cell anaphylatoxin receptors C3aR1 (p=0.04) and C5aR1 (p=0.03) in male mice with myocarditis by qRT-PCR during acute myocarditis (**Fig 6**). We also found that the complement regulator CR1/2 was not significantly increased in males with the BPA EPA reference dose during acute myocarditis (data not shown, p=0.05). Thus, even though mast cell numbers did not increase with BPA exposure in males (**Fig 4F**), increased expression of C3aR1 and C5aR1 suggest that BPA activates mast cells in males. In future studies we will examine whether there is evidence of mast cell degranulation in the heart by histology.

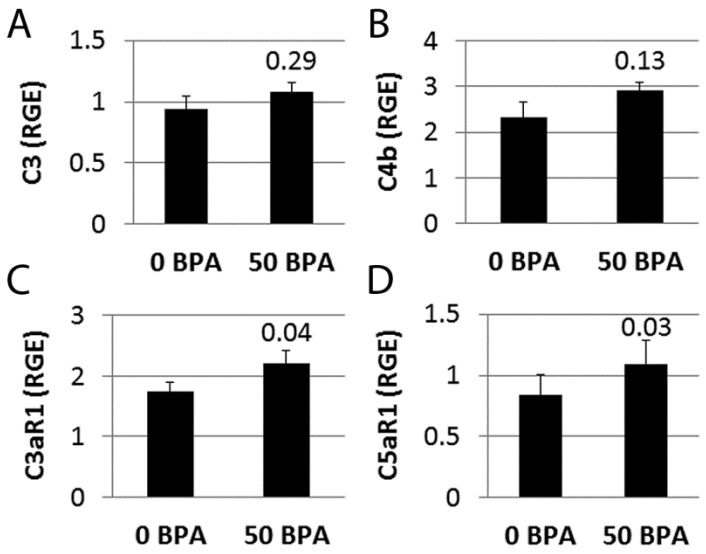

Figure 6. BPA exposure increases the mast cell anaphylatoxin receptors C3aR1 and C5aR1 during myocarditis in male mice housed in glass cages. Male BALB/c mice were given 0 (0 BPA) or 50 (50 BPA) μg BPA/kg BW in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. Soy-free bedding and food and glass cages and water bottles were used. BPA exposure continued from day 0 to 10 pi. Relative gene expression (RGE) of complement genes vs. the housekeeping control Hprt was examined for **A)** C3, **B)** C4b, **C)** C3aR1, and **D)** C5aR1 mRNA by qRT-PCR at day 10 pi comparing 0 to 50 μg BPA/kg BW groups.

Data show the mean +/-SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 10 mice/ group. 50 μg BPA/kg BW was obtained by dosing water with 250 μg BPA/L.

BPA exposure to male mice in glass cages increases IL-1R2 gene expression

TLR4/inflammasome activation is a key feature of the cardiac immune response in male BALB/c mice with CVB3 myocarditis (Fairweather 2003, Frisancho-Kiss 2006, Frisancho-Kiss 2007, Coronado 2012). Because myocarditis was increased with BPA exposure in male mice (**Fig 1**) and there was an increase in cardiac immune cells that can express TLR4 and the inflammasome (**Fig 4**), we examined TLR4 pathway-related genes using qRT-PCR. We found that BPA exposure at the EPA reference dose did not significantly alter most TLR4-related genes except for IL-1R2, which was significantly increased compared to control water (p=0.002) (**Fig 7D**). These data suggest that the primary mechanism where BPA increases myocarditis is not via the inflammasome. However, future experiments will need to determine whether active IL-1β is altered by BPA in males or if TLR4 pathway genes are altered by BPA at an earlier timepoint.

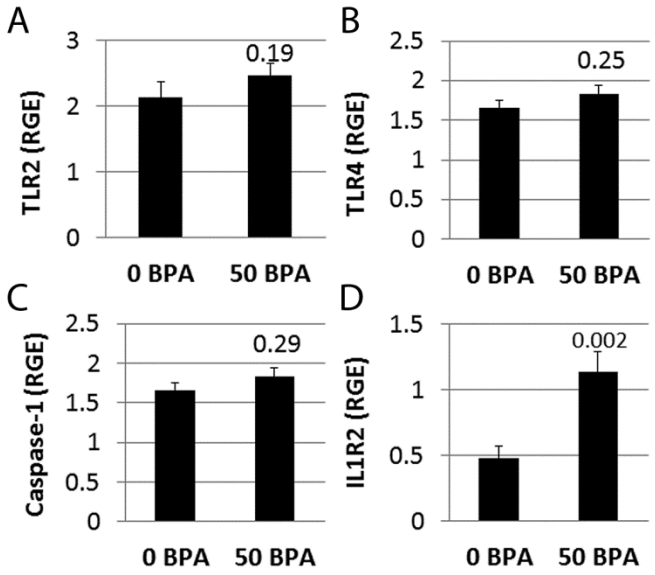

Figure 7. BPA exposure of male BALB/c mice increased IL-1R2 expression during myocarditis. Male BALB/c mice were given 0 (0 BPA) or 50 (50 BPA) μg BPA/kg BW in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. Soy-free bedding and food and glass cages and water bottles were used. BPA exposure continued from day 0 to 10 pi. Relative gene expression (RGE) of inflammasome-related genes vs. the housekeeping control Hprt for **A)** TLR2, **B)** TLR4, **C)** caspase-1, and **D) I**L-1R2 were examined in whole hearts by qRT-PCR at day 10 pi comparing 0 to 50 μg BPA/kg BW groups. Data show the mean +/-SEM using a two-tailed Student's *t* test with 10 mice/ group. 50 μg BPA/kg BW was obtained by dosing water with 250 μg BPA/L.

BPA exposure in male mice in glass cages increases the remodeling gene Timp1 during myocarditis

We showed previously that testosterone increases Timp1 expression in the heart during CVB3 myocarditis in male mice (Coronado 2012). Elevated cardiac TIMP-1 mRNA levels have been found to correlate with LV fibrosis in patients with chronic pressure overload (Heymans 2005), and elevated TIMP-1 levels in the sera predict the progression to heart failure in patients with coronary artery disease (Lubos 2005). Thus, elevated Timp1 contributes to cardiac remodeling in male BALB/c mice with CVB3 myocarditis, resulting in the progression to chronic cardiomyopathy. Because BPA exposure was found to increase myocarditis in males we examined whether BPA altered Timp1 levels. We found that BPA exposure at the EPA reference dose significantly increased Timp1 in the heart by qRT-PCR (p=0.03) (**Fig 8**), which could lead to increased remodeling in the heart of male mice with myocarditis.

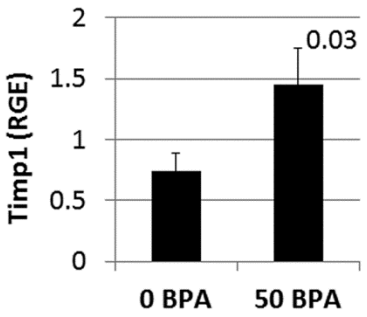

Figure 8. BPA exposure in male mice in glass cages increases Timp1 expression during myocarditis. Male BALB/c mice were given 0 (0 BPA) or 50 (50 BPA) μg BPA/kg BW in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. Soy-free bedding and food and glass cages and water bottles were used. BPA exposure continued from day 0 to 10 pi. Relative gene expression (RGE) of Timp1 vs. the housekeeping gene Hprt were analyzed in whole hearts by qRT-PCR at day 10 pi comparing 0 to 50 μg BPA/kg BW groups. Data show the mean +/-SEM using a two-tailed Mann-Whitney rank test with 10 mice/ group. 50 μg BPA/kg BW was obtained by dosing water with 250 μg BPA/L.

BPA exposure decreases ERRγ in males in glass cages with myocarditis, but has no significant effect on other sex hormone receptors

ERs and the AR are located on/in immune cells, cardiomyocytes, endothelial cells, and cardiac fibroblasts in both males and females (Regitz-Zagrosek 2008, Vitale 2009, Fairweather 2012A, Buskiewicz 2016). It is likely that the ratio of ERs to AR on/in immune cells is critical in sex hormone regulation of inflammation (Regitz-Zagrosek 2008, Vitale 2009, Fairweather 2012A, Buskiewicz 2016). However, this is a rapidly growing area of research with many questions that still remain. In this Chapter, we found that BPA exposure had no significant effect on the expression of ERα (p=0.51), ERβ (p=0.15), and AR (p=0.17), but there was a significant decrease in ERRγ (p=0.03) expression in the heart

compared to control water in male mice with CVB3 myocarditis by qRT-PCR (**Fig 9**). The role of ERRγ on/in inflammatory or resident cardiac cells is not yet known, but our data suggest that the EPA reference dose is able to significantly alter this ER in the heart during myocarditis in males.

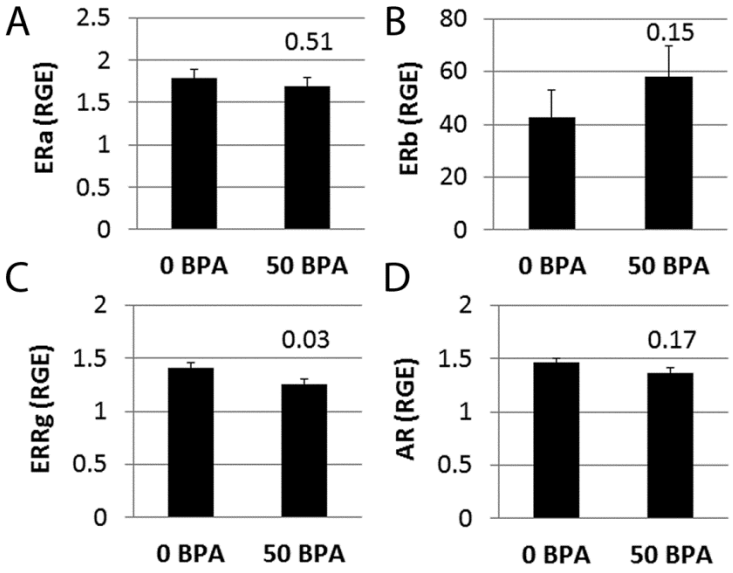

Figure 9. BPA exposure alters ERRγ in male BALB/c mice housed in glass caging. Male BALB/c mice were given 0 (0 BPA) or 50 (50 BPA) μg BPA/kg BW in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. Soy-free bedding and food and glass cages and water bottles were used. BPA exposure continued from day 0 to 10 pi. Relative gene expression (RGE) vs. Hprt controls of **A)** ERα **B)** ERβ **C)** ERRγ, and **D)** AR mRNA by qRT-PCR at day 10 pi comparing 0 to 50 μg BPA/kg BW groups. Data show the mean ±SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 10 mice/ group. 50 μg BPA/kg BW was obtained by dosing water with 250 μg BPA/L.

BPA increases VDR expression in the heart of males with myocarditis in glass cages

In **Chapter 3** we showed that VDR increases CVB3 myocarditis in male mice by increasing TLR4 pathway-related genes (**Chapter 3, Fig 3)**. Here we found that BPA exposure in male BALB/c mice significantly increased VDR expression in the heart by qRT-PCR (p=0.03) (**Fig 10**), but had no significant effect on Cyp enzymes that metabolize VitD to its active form (data not shown); Cyp2R1 (p=0.23), Cyp27B1 (p=0.31), and Cyp24A1 (p=0.24). This data suggests that at least one mechanism whereby BPA exposure can increase myocarditis is by elevating VDR expression in the heart. Plastic cages also had this effect on VDR expression in males.

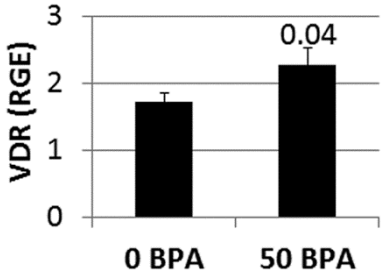

Figure 10. BPA increases VDR expression in the heart of males with myocarditis in glass cages. Male BALB/c mice were given 0 (0 BPA) or 50 (50 BPA) μg BPA/kg BW in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. Soy-free bedding and food and glass cages and water bottles were used. BPA exposure continued from day 0 to 10 pi. Relative gene expression (RGE) of VDR vs. the housekeeping gene Hprt were analyzed in whole hearts by qRT-PCR at day 10 pi comparing 0 to 50 μg BPA/kg BW groups. Data show the mean +/-SEM using a two-tailed Student's t test with 10 mice/ group. 50 μg BPA/kg BW was obtained by dosing water with 250 μg BPA/L.

Discussion

In this study we found that BPA exposure to adult male BALB/c mice housed in glass cages significantly increased myocarditis at day 10 pi at all BPA doses compared to control water (**Fig 1**). BPA exposure did not significantly alter viral levels in the heart of males (**Fig 3**), suggesting that increased myocarditis in males was not due to effects of BPA on viral replication. BPA increased the expression of nearly all infiltrating immune cell markers in male mice including macrophages, neutrophils, and T cells (**Fig 4**). ERRγ expression in the heart during CVB3 myocarditis was significantly lower after BPA exposure and VDR was significantly higher in males, but the other ERs were unchanged in males. Almost no studies exist for the effect of BPA on ERRγ. One study found that BPA works through ERRγ to induce developmental damage in a zebrafish model (Tohme 2014).

We found that many of the inflammatory pathways increased by VDR signaling in males in **Chapter 3** were increased by BPA treatment in males in glass cages (i.e., CD11b, complement anaphylactic receptors, Timp1). This suggests that BPA may be mediating its affect by altering VDR expression in the heart of males. However, more analysis of the effects of BPA in the pathogenesis of myocarditis in males is needed. The upregulation of C3aR1 and C5aR1 suggest mast cell activation, yet cKit levels were not significantly altered in the heart by BPA exposure. Thus, whether BPA is activating mast cells is unclear. In future experiments we need to examine the number and degranulation of mast cells in males using histology and/or flow cytometry. A recent cross-sectional study found that BPA exposure in men led to higher testosterone expression (Rochester 2013). Testosterone has been found to be responsible for the increase in CVB3 myocarditis in male mice compared to females (Coronado 2012). If BPA is leading to increased testosterone levels that could

explain, at least in part, the ability of BPA to increase myocarditis in males. Importantly, BPA has been found to affect the metabolism of Cyps involved in hormone metabolism and TSPO/ STAR which involves cholesterol transport into mitochondria for metabolism of sex steroids in macrophages and other cells (DeCoster 2012, Peretz 2013, Acconcia 2015). We have shown previously that TSPO is expressed only in CD11b+ immune cells compared to other immune cells during acute CVB3 myocarditis and that these CD11b+TSPO+ cells express TLR4 and release IL-1β and are responsible for the progression from acute to chronic myocarditis in male BALB/c mice (Onyimba 2011, Coronado 2012, Fairweather 2014). Other studies also suggested that BPA can bind to the AR, which could then directly activate the AR leading to increased disease in males (Molina-Molina 2013). We did not see an increase in AR expression in this study, but it may have been elevated during the innate immune response (Onyimba 2011), as cell surface receptors are known to cycle. Overall, our data show that BPA exposure is able to increase CVB3 myocarditis in male BALB/c mice using glass cages. In future studies we will need to assess the effect of BPA exposure at lower doses on myocarditis in males using glass cages and also examine the effect of BPA on males using plastic cages.

Chapter 8

Bisphenol A has no effect on myocarditis in female C57BL/6 mice housed in glass cages

Abstract

Since myocarditis is influenced by sex hormones, it is highly probable that endocrine disruptors, which interfere with natural hormones, will play a part in the progression of the disease. The human population is exposed to the endocrine disruptor BPA from plastics, such as water bottles, plastic food containers, and receipts, for example. To our knowledge no one has examined the role of endocrine disruptors like BPA on myocarditis in C57BL/6 (BL/6) mice. In this study we examined the role of BPA exposure on female BL/6 mice using glass cages and water bottles as well as soy-free food and bedding. We found that BPA exposure to BL/6 female mice had no significant effect on CVB3 myocarditis or immune cell populations in the heart. There was also no significant change in viral replication, body weight, or Th1/Th2/Th17-associated cytokines due to BPA exposure. We also assessed the effect of BPA exposure on complement and TLR4 signaling genes, two pathways that are known to increase CVB3 myocarditis, and found no significant change due to BPA exposure in female BL/6 mice. Lastly, we assessed if BPA could alter hormone expression in female BL/6 mice during CVB3 myocarditis and found no significant change in expression levels in the heart for any receptor. These results show that the effect of BPA is strain specific, which suggests that affects among people could vary by race.

Introduction

Human diseases including CVDs are known to differ in incidence and/or severity according to the race of the population under analysis (Fonseca 2016, McEvoy 2016, Zhang 2016). One way to assess race in experimental animal models is by using different strains of mice. Few studies have examined the effect of BPA on BL/6 mice. One study by Yan et al assessed the effect of BPA exposure on the immune response to *Leishmania* and found that inflammation was increased in BALB/c mice but was unchanged in BL/6 mice (Yan 2008).

We have shown previously that black background mice develop severe acute myocarditis after CVB3 infection with heart-passaged virus, but they do not progress to DCM (Fairweather 2001, Abston 2012A, Abston 2013). BL/6 mice respond to CVB3 infection with a strong IFN/Th1-type immune response that prevents progression to DCM (Fairweather 2004A, Abston 2012A, Abston 2013). Additionally, BL/6 mice have few mast cells in their spleen and peritoneum, and mast cells are critical in driving a mixed Th1/Th2-type immune response to CVB3 in male BALB/c mice that is both proinflammatory and profibrotic (Fairweather 2004A, Fairweather 2004B). Because of the different susceptibility to DCM following CVB3 myocarditis in BALB/c vs. BL/6 mice and because BPA had been found to activate mast cells in **Chapter 4**, we decided to examine whether BPA could alter CVB3 myocarditis in female BL/6 mice using glass cages and water bottles (and soy-free food and bedding). An additional reason we wanted to examine BL/6 mice was that ERα and ERβ knockout mice are available on a BL/6 background and if BPA altered myocarditis in female BL/6 mice, then we could use the knockout mice to explore mechanism without the need to backcross to a BALB/c background.

Results

BPA exposure has no effect on myocarditis in C57BL/6 female mice in glass cages

Myocarditis develops in response to CVB3 infection (our model of CVB3-induced myocarditis as well as other models of viral myocarditis) for all mouse strains that have been tested so far (i.e., BALB/c, C57BL/6, A/J, mixed BL background mice, etc.) (Fairweather 2001, Fairweather 2004A, Abston 2012A, Abston 2013). However, only BALB/c and A/J mice progress from myocarditis to chronic myocarditis and DCM (Fairweather 2001, Fairweather 2004A, Fairweather 2006, Coronado 2012). We showed previously that BL/6 mice develop a dominant Th1-type immune response that prevents remodeling and fibrosis, whereas A/J and BALB/c mice develop a dominant Th2 response that contributes to remodeling (Frisancho-Kiss 2007, Coronado 2012, Fairweather 2012B). Additionally, we previously published that BALB/c mice have abundant mast cells that contribute to a Th2-type immune response and release profibrotic cytokines and enzymes that drive remodeling in the heart (Fairweather 2004A, Fairweather 2004B). In contrast, BL/6 mice have very few mast cells.

To assess the effect of BPA on CVB3 myocarditis in BL/6 mice, 6-8 week old female BL/6 mice were given varying doses of BPA in water for 2 weeks prior to infection with CVB3 on day 0, as in previous Chapters. BPA treatment continued from day 0 to day 10 pi when mice were harvested. Soy-free food and bedding was used with glass cages and water bottles. Myocarditis was assessed histologically at day 10 pi during acute myocarditis by determining the % inflammation by H&E staining of heart sections. We found that BPA exposure did not significantly alter myocarditis at any dose in female BL/6 mice housed in glass cages (p=0.09) (**Fig 1**). Representative photos are shown in **Figure 2**.

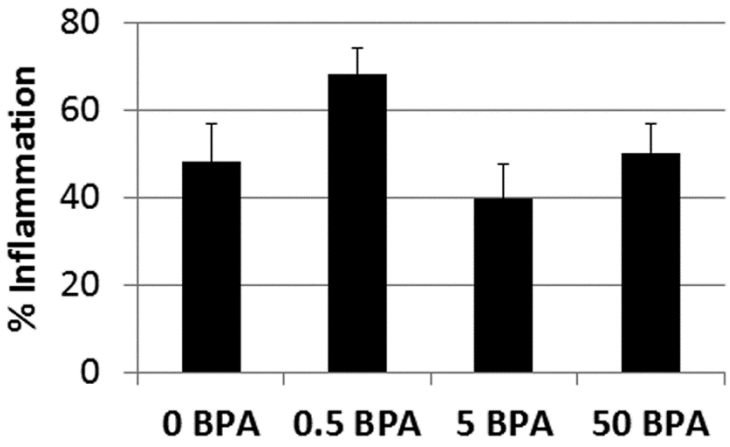

Figure 1. BPA has no effect on myocarditis at any BPA dose in female BL/6 mice in glass cages. Female BL/6 mice were given 0 (0 BPA), 0.5 (0.5 BPA), 5 (5 BPA), and 50 (50 BPA) μg BPA/kg BW in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and inflammation examined at day 10 pi, during peak myocarditis. BPA exposure continued from day 0 to 10 pi. Mice were housed using soy-free bedding and food. Myocarditis was assessed as the % inflammation in the heart using H&E compared to the overall size of the heart section by histology using an eyepiece grid. 0, 0.5, 5, and 50 μg BPA/kg BW estimated intakes were obtained by dosing water with 0, 2.5, 25 or 250 μg BPA/L, respectively. Data show the mean +/-SEM by one-way ANOVA for multiple comparisons (p=0.09), n=9-10 mice/ group.

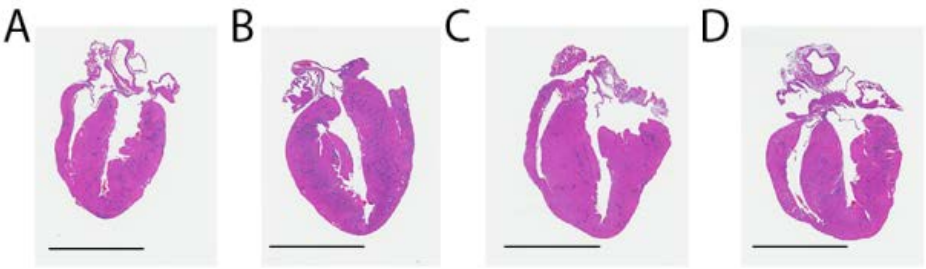

Figure 2. Representative photos of the effect of BPA exposure on myocarditis in BL/6 females in glass cages. Female BL/6 mice were given **A)** 0, **B)** 0.5, **C)** 5 , and **D)** 50 μg BPA/kg BW in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and inflammation examined at day 10 pi, during peak myocarditis. BPA exposure continued from day 0 to 10 pi. Mice

were housed using soy-free bedding and food. 0, 0.5, 5, and 50 μg BPA/kg BW estimated intakes were obtained by dosing water with 0, 2.5, 25 or 250 μg BPA/L, respectively. Inflammation in the heart was assessed using H&E stain where cardiac tissue appears pink and inflammation dark purple. The bar is 300μm in length.

BPA does not significantly alter viral gene expression or replication in the heart during myocarditis

We examined whether BPA exposure altered CVB3 replication in the heart during acute myocarditis. We used a primer/probe set directed to the CVB3 genome according to Antoniak et al (Antoniak 2013). We found that BPA had no significant effect on viral gene expression in the heart during acute myocarditis at day 10 pi by qRT-PCR (p=0.54) (**Fig 3A**) or viral replication by plaque assay (p=0.55) (**Fig 3B**).

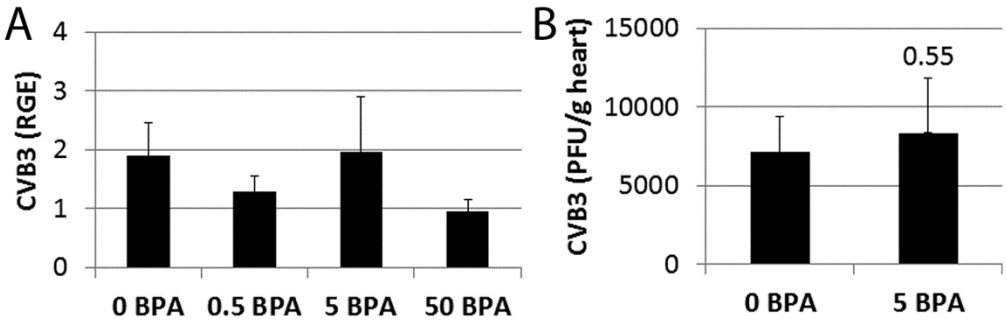

Figure 3. Viral gene expression in the heart was not effected by BPA treatment in BL/6 females in glass cages. Female BL/6 mice were given 0 (0 BPA), 0.5 (0.5 BPA), 5 (5 BPA), and 50 (50 BPA) μg BPA/kg BW in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and virus examined at day 10 pi, during peak myocarditis. BPA exposure continued from day 0 to 10 pi. Mice were housed using soy-free bedding and food. **A)** CVB3 levels in the heart were measured using qRT-PCR to determine relative gene expression (RGE) of viral genome compared to the housekeeping gene Hprt. Data show the mean +/-SEM by one-way ANOVA for multiple

comparisons (p=0.54), n=9-10 mice/ group. **B)** Viral replication was measured using plaque assay to determine PFU/g heart. Data show the mean +/-SEM by Student's t test (p=0.55), n=10 mice/ group. 0, 0.5, 5, and 50 μg BPA/kg BW estimated intakes were obtained by dosing water with 0, 2.5, 25 or 250 μg BPA/L, respectively.

BPA exposure does not affect body weight of BL/6 female mice in glass cages

BPA has been found to increase type II diabetes (Bodin 2014, Rezg 2014). Body weight was assessed to determine if BPA dosing in the water caused a change in body weight in BL/6 female mice housed in glass cages. We found that BPA had no significant effect on body weight in the heart during acute myocarditis at day 10 pi (p=0.07) (**Fig 4**).

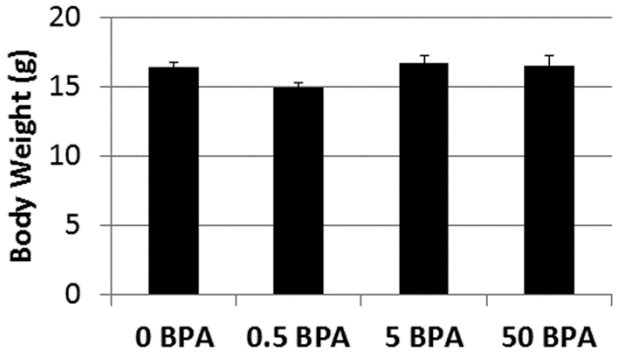

Figure 4. BPA did not affect body weight during acute myocarditis in BL/6 female mice in glass cages. Female BL/6 mice were given 0 (0 BPA), 0.5 (0.5 BPA), 5 (5 BPA), and 50 (50 BPA) μg BPA/kg BW in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and body weight examined at day 10 pi, during peak myocarditis. BPA exposure continued from day 0 to 10 pi. Mice were housed using soy-free bedding and food. Body weight was determined at day 10 pi. Data are shown as the mean +/-SEM using one-way ANOVA for multiple comparisons with 9-10 mice/ group (p=0.07). 0, 0.5, 5, and 50 μg BPA/kg BW were obtained by dosing water with 0, 2.5, 25 or 250 μg BPA/L, respectively.

BPA exposure has no effect on markers for individual immune cell populations in the heart of BL/6 females in glass cages

Although we saw no difference in myocarditis by histology (**Fig 1**), it is possible for cell populations to differ/shift based on treatments and so we examined markers for major immune cell populations in the heart by qRT-PCR in BL/6 females housed in glass cages and exposed to various doses of BPA. We found BPA exposure had no effect on CD45 (p=0.86), CD11b (p=0.15), or cKit (p=0.31) in BL/6 female mice housed in glass cages (**Fig 5**). We also saw no significant difference in CD3 (p=0.58), CD4 (p=0.53), CD8 (p=0.42) or CD19 (p=0.83) expression (**Fig 6**). These results are consistent with the histology data in **Figure 1**.

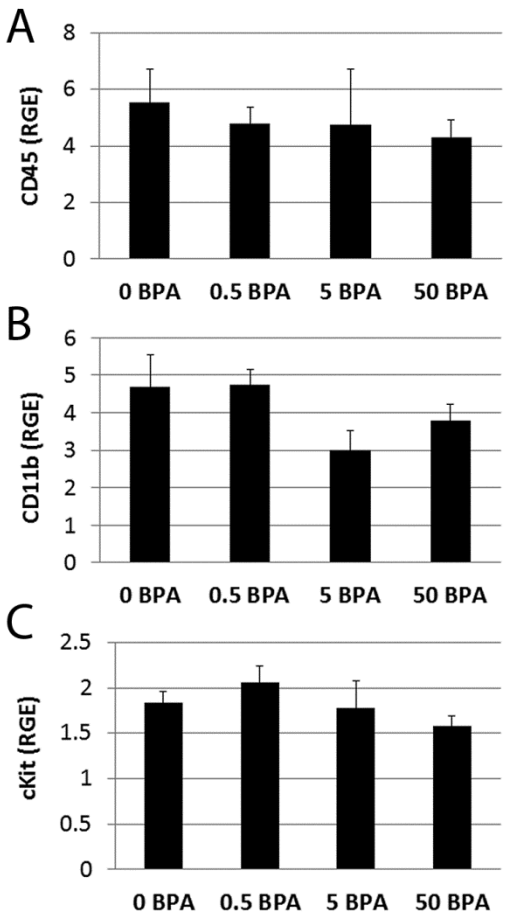

Figure 5. CD45, CD11b and cKit expression were not altered by BPA exposure in BL/6 female mice in glass cages. Female BL/6 mice were given 0 (0 BPA), 0.5 (0.5 BPA), 5 (5 BPA), and 50 (50 BPA) μg BPA/kg BW in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and immune cells examined at day 10 pi, during peak myocarditis. BPA exposure continued from day 0 to 10 pi. Mice were housed using soy-free bedding and food. Immune cell populations from the heart were examined using qRT-PCR for relative gene expression (RGE) of the gene of interest vs. the housekeeping gene Hprt for **A)** CD45 (total immune cells) (p=0.86), **B)** CD11b (includes macrophages, neutrophils, mast cells and some dendritic cells) (p=0.15), and **C)** cKit (mast cells) (p=0.31). Data are shown as the mean +/-SEM using one way ANOVA for multiple comparisons with 7-10 mice/ group. 0, 0.5, 5, and 50 μg BPA/kg BW were obtained by dosing water with 0, 2.5, 25 or 250 μg BPA/L, respectively.

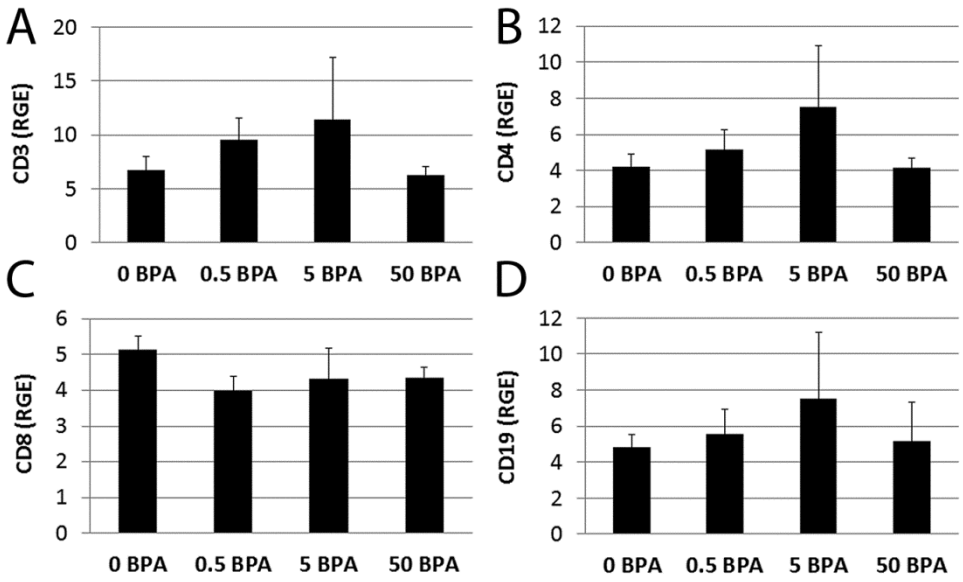

Figure 6. CD3, CD4, CD8 and CD19 expression were not altered by BPA exposure in BL/6 female mice in glass cages. Female BL/6 mice were given 0 (0 BPA), 0.5 (0.5 BPA), 5 (5 BPA), and 50 (50 BPA) μg BPA/kg BW in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and immune cells examined at day 10 pi, during peak myocarditis. BPA exposure continued from day 0 to 10 pi. Mice were housed using soy-free bedding and food. Immune cell populations from the heart were examined using qRT-PCR for relative gene expression (RGE) of the gene of interest vs. the housekeeping gene Hprt for **A)** CD3 (total T cells) (p=0.58), **B)** CD4 (CD4+ T cells) (p=0.53), **C)** CD8 (CD8+ T cells) (p=0.42), and **D)** CD19 (B cells) (p=0.83). Data are shown as the mean +/-SEM using one way ANOVA for multiple comparisons with 9-10 mice/ group. 0, 0.5, 5, and 50 μg BPA/kg BW were obtained by dosing water with 0, 2.5, 25 or 250 μg BPA/L, respectively.

BPA exposure had no influence on cardiac cytokine levels in BL/6 female mice housed in glass cages

We examined whether IFNγ, IL-4, IL-17A, and IL-1β cytokine levels were altered in the heart during acute CVB3 myocarditis following 5 μg BPA /kg BW exposure in BL/6

females housed in glass cages. Changes in IFNγ, IL-4, and IL-17A are often used to indicate whether a Th1, Th2 or Th17 response is dominant, respectively (Fairweather 2004C, Zhu 2010). We found that IFNγ (p=0.05), IL-4γ (p=0.35), IL-17 (p=0.61), and IL-1β (p=0.15) were not significantly changed due to BPA exposure in BL/6 females during acute myocarditis (**Fig 7**).

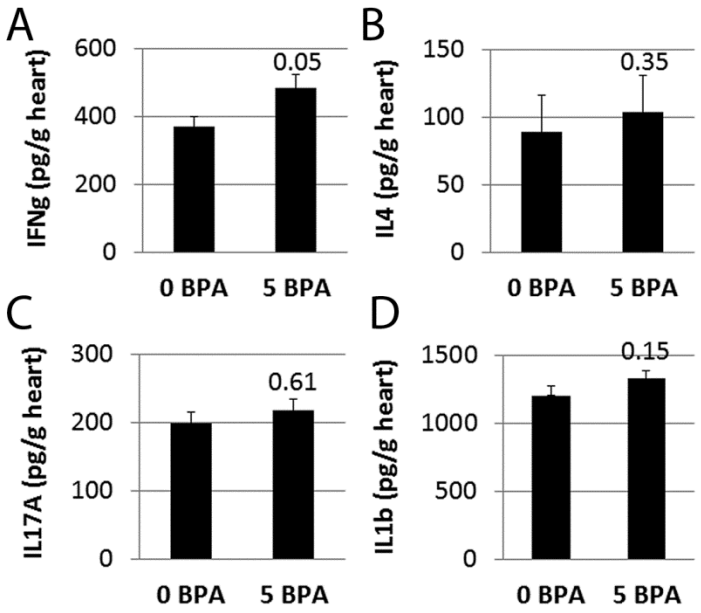

Figure 7. Cardiac cytokine levels were not altered by BPA exposure during CVB3 myocarditis. Female BL/6 mice were given 0 (0 BPA) or 5 (5 BPA) μg BPA/kg BW in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and cytokines examined from homogenized heart supernatant at day 10 pi, during peak myocarditis. BPA exposure continued from day 0 to 10 pi. Mice were housed using soy-free bedding and food. The supernatant was used to measure **A)** IFNγ, **B)** IL-4, **C)** IL-17A, and **D)** IL-1β levels in the heart by ELISA. Data show the mean +/-SEM using a two-tailed Mann-Whitney rank test with 28-29 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPA exposure decreases C3aR1 at high human relevant dose, but has no effect on other complement components in BL/6 females in glass cages

Because of the important role of complement components in the induction of CVB3 myocarditis in BALB/c and A/J mice (Fairweather 2006) and because their levels were affected by BPA in **Chapter 4**, we examined whether BPA exposure altered complement components in the heart of female BL/6 mice housed in glass cages with myocarditis (**Fig 8**). We found that BPA exposure significantly decreased C3aR1 (p=0.01) (**Fig 8C**), but had no significant effect on other complement components including C3 (p=0.85), C4b (p=0.16), C5aR1 (p=0.15), CD11b/CR3 (p=0.15), or CR1/2 (p=0.79) (**Fig 8**).

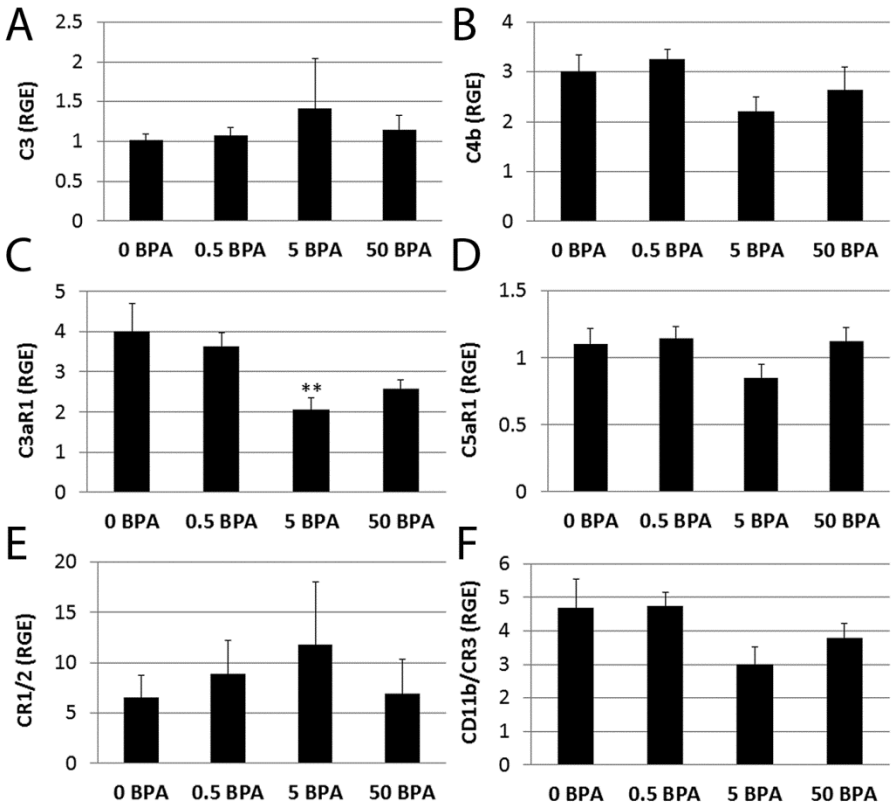

Figure 8. Effect of BPA exposure on complement components in BL/6 female in glass during myocarditis. Female BL/6 mice were given 0 (0 BPA), 0.5 (0.5 BPA), 5 (5 BPA), and 50 (50 BPA) μg BPA/kg BW in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0

and complement components examined at day 10 pi, during peak myocarditis. BPA exposure continued from day 0 to 10 pi. Mice were housed using soy-free bedding and food. Relative gene expression (RGE) of complement genes vs. the housekeeping control Hprt was examined for **A)** C3, **B)** C4b, **C)** C3aR1, **D)** C5aR1, **E)** CR1/2, and **F)** CR3/CD11b mRNA by qRT-PCR at day 10 pi comparing 0 μg BPA/kg BW to increasing BPA estimated intake groups. Data are shown as the mean +/-SEM using one-way ANOVA for multiple comparisons with 9-10 mice/ group (**Post hoc test, p<0.01). 0, 0.5, 5, and 50 μg BPA/kg BW were obtained by dosing water with 0, 2.5, 25 or 250 μg BPA/L, respectively.

BPA exposure decreases TLR4 expression at EPA reference dose, but has no effect on caspase-1 in the heart of female BL/6 in glass cages during myocarditis

When we examined components of TLR4 signaling, we found that BPA exposure significantly decreased TLR4 expression at the highest BPA dose (i.e., EPA reference dose) (one-way ANOVA, p<0.0001, Student's t test p=0.000003) (**Fig 9A**), but did not have a significantly affect on caspase-1 (p=0.39) (**Fig 9B**) or IL-1β (p=0.15) (**Fig 7D**) in the heart of female BL/6 mice in glass cages during acute myocarditis.

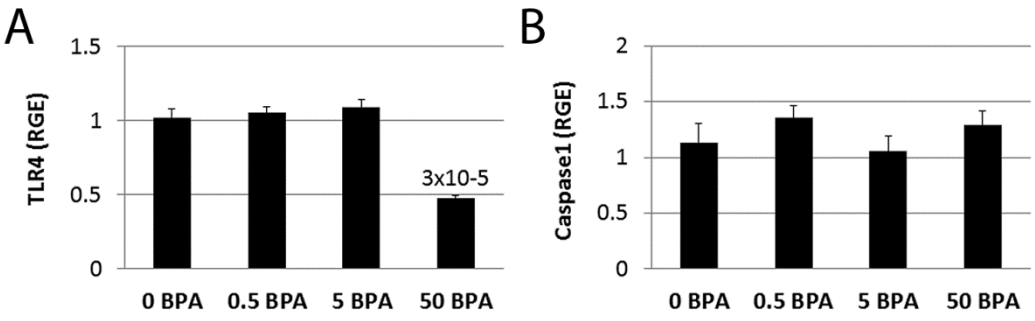

Figure 9. EPA reference dose of BPA decreases TLR4 in the heart of BL/6 female mice in glass cages during myocarditis. Female BL/6 mice were given 0 (0 BPA), 0.5 (0.5 BPA), 5 (5 BPA), and 50 (50 BPA) μg BPA/kg BW in drinking water for 2 weeks and then injected ip with 10^3 PFU of

CVB3 ip on day 0 and complement components examined at day 10 pi, during peak myocarditis. BPA exposure continued from day 0 to 10 pi. Mice were housed using soy-free bedding and food. Relative gene expression (RGE) of inflammasome genes vs. the housekeeping control Hprt for **A)** TLR4 and **B)** caspase-1 were examined in whole hearts by qRT-PCR at day 10 pi comparing control water to BPA treatment Data are shown as the mean +/-SEM using one-way ANOVA for multiple comparisons with 9-10 mice/ group for gene expression. 0, 0.5, 5, and 50 μg BPA/kg BW were obtained by dosing water with 0, 2.5, 25 or 250 μg BPA/L, respectively.

BPA exposure has no effect on sex hormone receptor expression in the heart of BL/6 females in glass cages

When we examined ERs and AR expression in the heart by qRT-PCR of BL/6 female mice with myocarditis that had been housed in glass cages, we found that BPA exposure had no significant effect on hormone expression of ERα (p=0.37), ERβ (p=0.29), ERRγ (p=0.73), or AR (p=0.90) in the heart during CVB3 myocarditis (**Fig 10**).

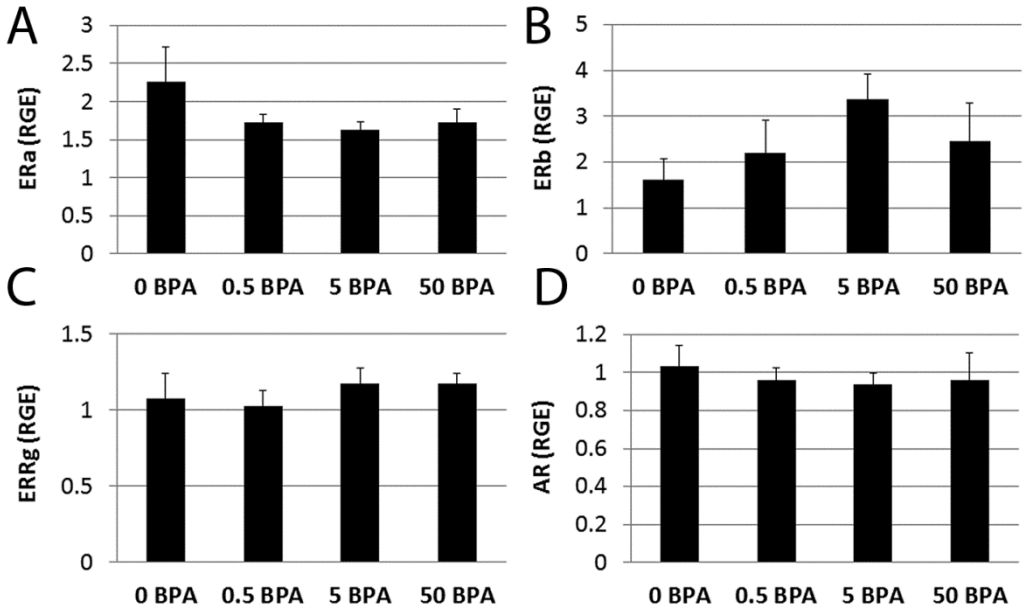

Figure 10. BPA exposure has no effect on sex hormone receptor expression in the heart of BL/6 females in glass cages. Female BL/6 mice were given 0 (0 BPA), 0.5 (0.5 BPA), 5 (5 BPA), and 50 (50 BPA) μg BPA/kg BW in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and complement components examined at day 10 pi, during peak myocarditis. BPA exposure continued from day 0 to 10 pi. Mice were housed using soy-free bedding and food. Relative gene expression (RGE) vs. Hprt controls of **A)** ERα, **B)** ERβ **C)** ERRγ and **D)** AR mRNA by qRT-PCR at day 10 pi. Data show the mean +/-SEM, *n*=7-10/group. Significant differences were calculated using one-way ANOVA for multiple comparisons. 0, 0.5, 5, and 50 μg BPA/kg BW were obtained by dosing water with 0, 2.5, 25 or 250 μg BPA/L, respectively.

Free BPA and BPA-glucuronide levels were not significantly altered between 0 and 25 μg BPA/L water doses in female BL/6 mice in glass cages

Free BPA and BPA-glucuronide levels were measured by LC-MS/MS in the urine of female BL/6 mice exposed to control or BPA-treated water after infection. No significant differences were found in BPA levels between BPA-treated water compared to control water without BPA: free BPA (p=0.73) and BPA-glucuronide (p=0.96) (**Fig 11**). The inability to detect a significant difference in BPA levels in the urine of BL/6 mice assessed by LC-MS/MS could have arisen from a number of situations. Another cause could be due to the urine being stored for a year in plastic Eppendorf tubes, which may have leached BPA and altered the levels in the samples (Vandenberg 2009). In future studies we will collect water, urine and blood to test BPA levels in glass containers in order to prevent potential BPA leaching during storage and analysis.

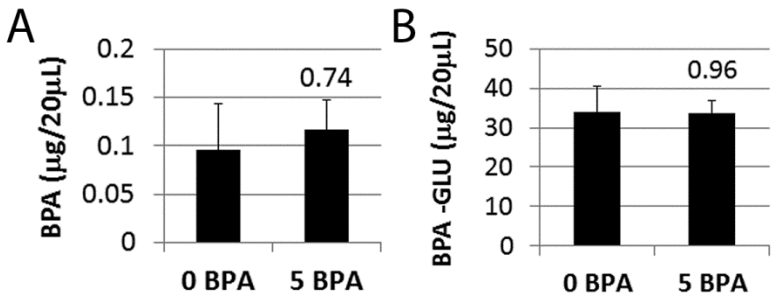

Figure 11. BPA concentration in urine of female BL/6 mice was unchanged by BPA dose when assessed by LC-MS/MS. Female BL/6 mice were given 0 (0 BPA) or 5 (5 BPA) µg BPA/kg BW in drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0. BPA exposure continued from day 0 to 10 pi. Mice were housed using soy-free bedding and food using glass cages and water bottles. **A)** Free BPA and **B)** BPA-glucuronide levels were assessed in the urine of mice. Urine was collected from individual mice on day 0, 3, 7, 10 pi and samples were pooled for each mouse. Samples were sent to our collaborators at University of Kentucky for analysis of BPA levels by LC-MS/MS. Significance was calculated using Mann-Whitney rank test comparing BPA exposure to control water group. Data show the mean ±SEM with 3 mice/ group. 5 µg BPA/kg BW was obtained by dosing water with 25 µg BPA/L.

Discussion

In this study we found that BPA exposure to adult female BL/6 mice housed in glass cages (and using soy-free bedding and food) had no significant effect on myocarditis at day 10 pi. Using plaque assay and qRT-PCR to determine viral replication in the pancreas and and heart, respectively, virus was not altered by BPA exposure. BPA did not alter immune cell populations, body weight, cytokines, TLR4 pathway genes or complement genes in female BL/6 mice. ERα, ERβ, AR and ERRγ levels were unchanged according to qRT-PCR after BPA exposure compared to control water. Thus, BPA exposure in female BL/6 mice had no significant effect on acute myocarditis. Both our study and Yan et al found that the effect of BPA exposure on inflammation after infection was only observed in BALB/c mice (Yan 2008). Based on our finding that a central effect of BPA exposure in female BALB/c mice was to increase mast cell numbers and degranulation in **Chapter 4 and 5**, this suggests that the low numbers of mast cells present in BL/6 mice may prevent immune cell activation via this route. We have published previously that mast cells are the first antigen presenting cells to respond to CVB3 infection in our model in the spleen and peritoneum compared to dendritic cells, monocyte/macrophages, and B cells (Frisancho-Kiss 2006A). Thus, it is possible that BPA exposure does not affect BL/6 females due to their lack of or low numbers of mast cells. This idea will need to be verified in future studies.

We assessed urine levels of free BPA by LC-MS/MS to determine if less active BPA was present in the urine of BL/6 mice. The average BPA concentration in BL/6 female mice was 0.0012 μg/L, and this level was similar to that found by LC-MS/MS in BALB/c female mice (0.0014 μg/L). Therefore, BPA concentration in the body does not appear to explain why BPA exposure had no significant effect on myocarditis in female BL/6 mice.

Chapter 9

Bisphenol S, a replacement for bisphenol A in plastics, increases myocarditis in female BALB/c mice using glass cages

Abstract

Myocarditis is an inflammatory heart disease that leads to DCM and heart failure. Sex hormones play a vital role in development of myocarditis with testosterone driving disease in males. Since myocarditis is influenced by sex hormones, it is highly probable that endocrine disruptors, which interfere with natural hormones, will play a part in the progression of the disease. The human population is exposed to the endocrine disruptor BPS from plastics such as water bottles and plastic food containers, for example. BPS is the plastic that has replaced BPA in many water and food containers. Very few studies have assessed the effect of BPS on disease states, and to our knowledge no one has examined the role of BPS on myocarditis. Glass cages and water bottles as well as soy-free food and bedding were used in these experiments in order to assess the effect of BPS exposure on female BALB/c mice without the confounding effect of leaching from plastic caging. The dose we used was equivalent to the BPA high human relevant dose of 25 μg BPA/L (5 μg BPA/kg BW) and was 27.5 μg BPS/L, which has an estimated intake exposure of 5.5 μg BPS/kg BW. We found that this dose of BPS significantly increased myocarditis in female BALB/c mice compared to control water. We also found that BPS had no effect on viral levels in the heart. We found that BPS exposure increased markers for CD4 and CD8 T cells and mast cells (cKit), which correlates with increased inflammation. BPS significantly increased ERα expression in the heart, which is known to be important in T cell proliferation. The increase in myocarditis was associated with an increase in M1 macrophage markers and TLR2, caspase-1, and IL-1R2 expression. Thus, BPS appears to increase CVB3 myocarditis in BALB/c females using very similar mechanisms as we found for BPA in plastic cages. However, in these studies with BPS we used glass cages so there was no confounding influence of plastics leaching from the cages.

Introduction

Although BPA has been mandated to be removed from baby bottles and sippy cups and voluntarily removed by manufactures from other products such as plastic water bottles, it has been replaced with another endocrine disruptor- BPS. Therefore the study of BPS is extremely important because as BPA is removed from more and more plastic materials over time it is likely to be replaced with BPS.

BPS can bind to ERα and ERβ, and is considered more estrogenic than BPA (Rochester 2015). When estradiol is bound to ERα, BPA can remove estradiol from the binding site whereas BPS only inhibited about 80% of the binding of estradiol (Molina-Molina 2013). Additionally, BPA is able to activate both ERα and ERβ, whereas BPS activates ERβ more than ERα (Molina-Molina 2013). BPS is also thought to bind to the AR receptor as an agonist at a very low binding level of around 20% of that of testosterone (Molina-Molina 2013). However, BPA can also act as an antagonist to the AR, while BPS does not (Molina-Molina 2013). One study assessed the effect of BPS on CVD and found that BPS exposure was able to increase heart rate, arrhythmias, induced calcium handling changes via ERβ in females, but not males- similar to BPA (Gao 2015). No studies have assessed the effect of BPS on CVB3 myocarditis.

Results

BPS increases myocarditis in female BALB/c mice at high human relevant dose (5.5μg/kg)

Studies have found that BPA increases cardiac, autoimmune and inflammatory diseases such as cardiac arrhythmias (Orton 2004, Weinstock-Guttman 2003, Kendziorski 2012, Melzer 2012, Shankar 2012, Gao 2014, O'Brien 2014, Jochmanova 2015, Ranciere 2015, Han 2016, He 2016). However, few studies have assessed the effect of BPS exposure in animal models (Gao 2015), and there have been no studies on the effect of BPS exposure on inflammatory diseases including myocarditis. To assess the effect of BPS exposure on CVB3 myocarditis, 6-8 week old female BALB/c mice were given varying doses of BPS in their drinking water for two weeks prior to ip infection with CVB3 on day 0. BPS exposure was continued until day 10 harvest. Glass cages and water bottles with soy-free bedding and food were used for all experiments in this Chapter. Because we had found that a dose of 5 μg BPA/kg BW had significantly increased myocarditis in plastic cages (**Chapter 4, Fig 2**) (and we are not aware of any studies that estimate a low-dose or "safe" lifetime dose for BPS), we examined the effect of an equivalent dose of BPS on myocarditis in this Chapter. We determined that 27.5 μg BPS/L water was equivalent to an estimated intake of 5.5 μg BPS/kg BW based on an analytical calculation of Molarity.

The effect of BPS exposure on CVB3 myocarditis was assessed histologically at day 10 pi during acute myocarditis. BPS exposure in drinking water was continued from day 0 until harvest at day 10 pi. We found that a 27.5 μg BPS/L exposure in drinking water significantly increased myocarditis in BALB/c female mice housed in glass cages compared to control water containing no BPS (p=0.03) (**Fig 1**). As expected, female BALB/c mice with control water developed a low level of myocarditis (i.e., 20%) at day 10 pi (Frisancho-Kiss

2007, Coronado 2012). Representative photos of the effect of BPS exposure on CVB3 myocarditis in BALB/c females are shown in **Figure 2**.

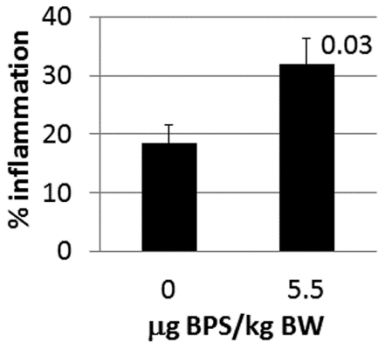

Figure 1. BPS exposure increases myocarditis in BALB/c female mice at high human relevant dose (5.5 µg BPS/kg BW). Female BALB/c mice were given 0 or 5.5 µg BPS/kg BW bisphenol S (BPS) in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and inflammation examined at day 10 pi, during peak myocarditis. Myocarditis was assessed as the % inflammation in the heart compared to the overall size of the heart section by histology using an eyepiece grid. Data are shown as the mean +/-SEM from 10 mice/ group. Significance was determined using a two-tailed Student's *t* test. 5.5 µg BPs/kg BW was obtained by dosing water with 27.5 µg BPA/L.

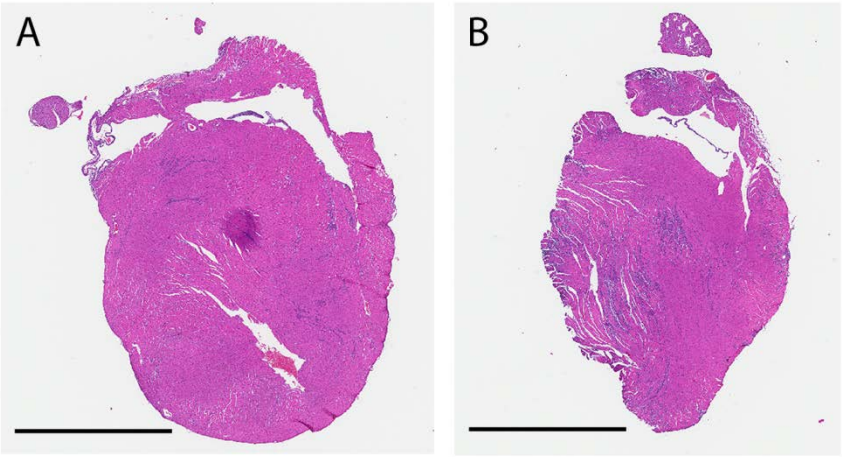

Figure 2. Representative inflammation after BPS exposure in BALB/c female mice during myocarditis. Female BALB/c mice were given **A)** 0 or **B)** 5.5 μg BPS/kg BW bisphenol S (BPS) in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and inflammation examined at day 10 pi, during peak myocarditis. Representative H&E photos of myocardial inflammation. Pink represents cardiac tissue and purple is areas of myocardial inflammation. Bar is 2mm in length. 5.5 μg BPS/kg BW was obtained by dosing water with 27.5 μg BPS/L.

BPS does not significantly alter viral gene expression in the heart during myocarditis

We examined whether BPS exposure altered CVB3 levels in the heart during acute myocarditis. We used a primer/probe set directed to the CVB3 genome according to Antoniak et al (Antoniak 2013). We found that BPS had no significant effect on viral expression levels in the heart during acute myocarditis at day 10 pi by qRT-PCR (p=0.11) (**Fig 3**). Thus, increased myocardial inflammation following BPS exposure was not due to increased viral replication during acute myocarditis.

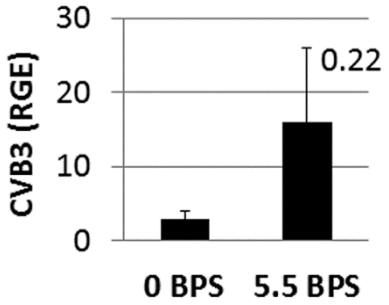

Figure 3. Viral gene expression in the heart was not effected by BPS treatment. Female BALB/c mice were given 0 (0 BPS) or 5.5 (5.5 BPS) μg BPS/kg BW in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and inflammation examined at day 10 pi, during peak myocarditis. CVB3 levels in the heart were measured using qRT-PCR to determine relative gene expression (RGE) of CVB3 genomes compared to the housekeeping gene Hprt. Data are shown as the mean +/-SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 9-10 mice/ group. 5.5 μg BPS/kg BW was obtained by dosing water with 27.5 μg BPS/L.

BPS exposure does not affect body weight

Recently BPA has been associated with an increased risk of type II diabetes (Bodin 2014, Rezg 2014) no studies on BPS. We examined whether BPS altered the body weight of female BALB/c mice and found that BPS exposure had no significant effect on body weight during acute myocarditis at day 10 pi (p=0.59) (**Fig 4**). These data suggest that the role of BPS in increasing myocarditis is not by increasing body weight.

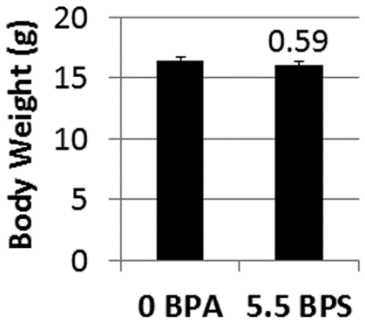

Figure 4. Body weight was not altered due to exposure to BPS in female BALB/c mice in glass cages. Female BALB/c mice were given 0 (0 BPS) or 5.5 (5.5 BPS) μg BPS/kg BW in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and body weight examined at day 10 pi, during peak myocarditis. Body weight was determined at day 10 pi. Data are shown as the mean +/-SEM using a two-tailed Student's *t* test with 9-10 mice/ group. 5.5 μg BPS/kg BW was obtained by dosing water with 27.5 μg BPS/L.

BPS exposure increases expression of immune cell markers including mast cells in female BALB/c mice in glass cages

The effect of BPS exposure on markers of specific cardiac immune cells during acute myocarditis at day 10 pi was assessed using qRT-PCR. We found that 5.5μg BPS/ kg BW exposure to BALB/c female mice housed in glass cages significantly increased CD45 (p=0.02) and cKit (mast cells, p=0.0005) in the heart compared to 0 BPS control water during CVB3 myocarditis (**Fig 5**). GR1 (p=0.05), CD11b (p=0.08), and F4/80 (p=0.05) were borderline significantly increased (**Fig 5**). CD14 expression was not significantly different between 5.5μg BPS and 0 BPS control water (p=0.15) (**Fig 5E**). The increase in cKit/ mast cells following BPS exposure in this Chapter was also observed in female BALB/c mice in plastic cages following BPA exposure (**Chapter 4**, **Fig 10**). The borderline increase in many of these immune cell populations indicates that the number of mice/ group needs to be increased in future experiments. Future experiments will also examine mast cell numbers and degranulation using histology and/or flow cytometry to confirm that the increase in cKit is due to mast cells.

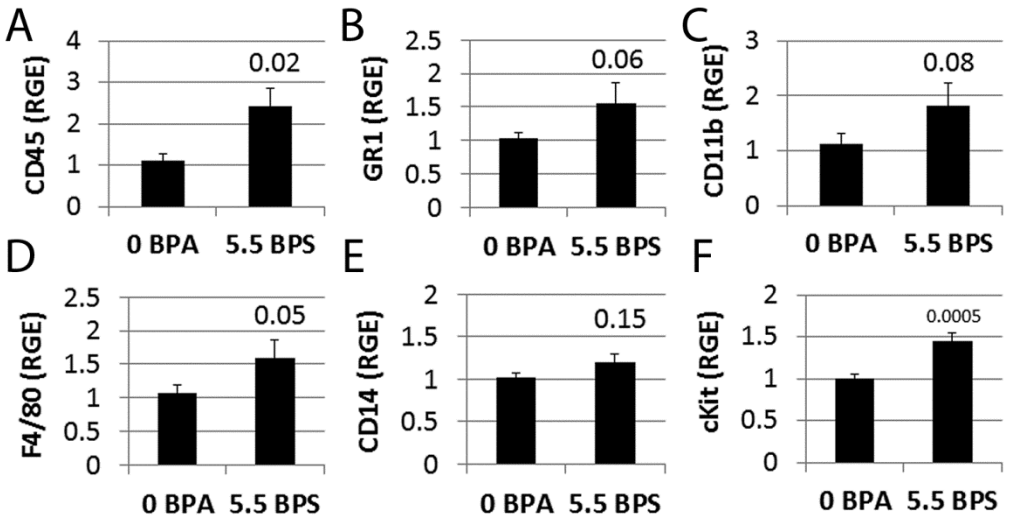

Figure 5. CD45 and cKit are significantly increased by BPS exposure in BALB/c female mice in glass cages. Female BALB/c mice were given given 0 (0 BPS) or 5.5 (5.5 BPS) μg BPS /kg BW in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. Immune cell populations from the heart were examined using qRT-PCR for relative gene expression (RGE) of the gene of interest vs. the housekeeping gene Hprt for **A)** CD45 (total immune cells), **B)** GR1 (neutrophils), **C)** CD11b (includes macrophages, neutrophils, mast cells and some dendritic cells), **D)** F4/80 (macrophages), **E)** CD14 (part of TLR4 signaling on mast cells and macrophages), and **F)** cKit (mast cells or stem cells). Data are shown as the mean +/- SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 9-10 mice/ group. 5.5 μg BPS/kg BW was obtained by dosing water with 27.5 μg BPS/L.

BPS exposure increases markers of T cells in female BALB/c mice in glass cages

The effect of BPS exposure on markers for T cells during acute myocarditis at day 10 pi was assessed using qRT-PCR. We found that 5.5μg BPS/ kg BW exposure to BALB/c female mice housed in glass cages significantly increased expression of CD4 (p=0.04) and CD8 (p=0.03) in the heart compared to 0 BPS control water during CVB3 myocarditis (**Fig**

6), indicating an increase in CD4+ and CD8+ T cells. CD3 expression was borderline significantly increased (p=0.06) (**Fig 6A**). We also examined Foxp3 expression (Treg cell marker), but found no significant difference between 5.5μg BPS and 0 BPS control water for this immune cell marker (p=0.10) (**Fig 6D**). The increase in T cell markers by qRT-PCR during acute myocarditis following BPS exposure is consistent with the increased inflammation observed with histology (**Fig 1**). The number of mice/ group also needs to be increased for the T cell analysis and cells analyzed using flow cytometry.

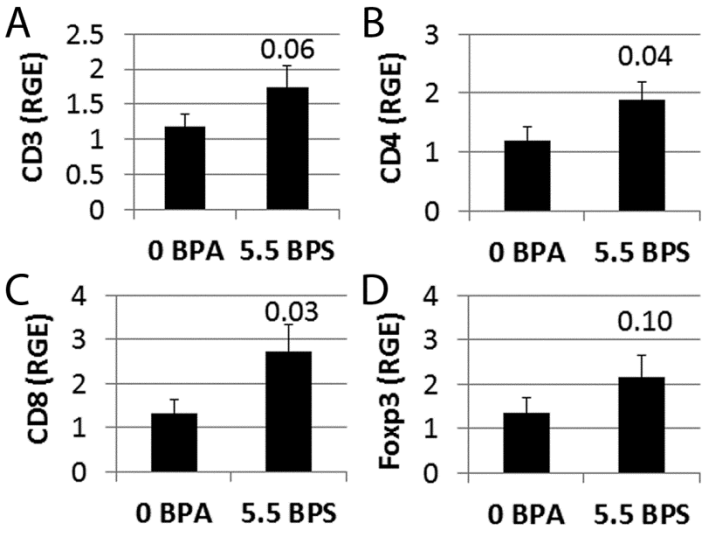

Figure 6. T cells are significantly increased by BPS exposure in BALB/c female mice in glass cages. Female BALB/c mice were given 0 (0 BPS) or 5.5 (5.5 BPS) μg BPS /kg BW in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. Immune cell populations from the heart were examined using qRT-PCR for relative gene expression (RGE) of the gene of interest vs. the housekeeping gene Hprt for **A)** CD3 (total T cells), **B)** CD4 (CD4+ T cells), **C)** CD8 (CD8+ T cells), and **D)** Foxp3 (Treg). Data are shown as the mean +/-SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 9-10 mice/ group. 5.5 μg BPS/kg BW was obtained by dosing water with 27.5 μg BPS/L.

BPS exposure increases markers of classically activated M1 macrophages

Markers of M2 macrophages in mice include arginase-1 (Arg1) and Ym1 (eosinophilic protein from chitinase family), while M1 markers include Cxcl9 and Cxcl10 (Siracusa 2008, Fairweather 2009, Abston 2012A). Analysis of M1 vs. M2 macrophage markers using qRT-PCR revealed a borderline increase for the M1 marker Cxcl9 (p=0.05) and a significant increase in the M1 marker Cxcl10 (p=0.03), but no significant increase in M2 markers arginase-1 (p=0.38) and Ym1 (p=0.23) with BPS exposure compared to water controls (**Fig 7**). The data suggest that BPS increases a Th1-type immune response that is associated with elevated classically activated M1 macrophage responses. The data also suggest that we need to increase the number of mice/ group, which we will do in future experiments, and that we need to conduct ELISA to determine Th1 vs. Th2 cytokine profiles that are usually associated with M1 vs. M2 skewing.

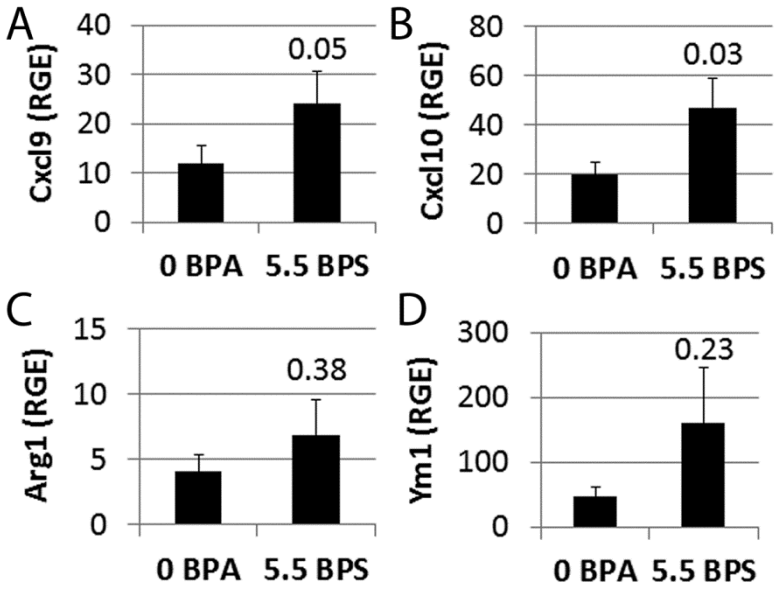

Figure 7. BPS exposure increases markers of classically activated M1 macrophages. Female BALB/c mice were given 0 (0 BPS) or 5.5 (5.5 BPS) μg BPS /kg BW in their drinking water for 2

weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. Relative gene expression (RGE) of **A)** M1 macrophage markers (Cxcl9, Cxcl10) and **B)** M2 macrophage markers (Arg1, Ym1) vs. Hprt controls were assessed in whole hearts using qRT-PCR at day 10 pi comparing 0 and 5 μg BPS/kg BW groups. Data show the mean ±SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 9-10 mice/ group. 5.5 μg BPS/kg BW was obtained by dosing water with 27.5 μg BPS/L.

BPS exposure does not alter expression of complement components in female BALB/c mice in glass cages

Complement components play an important role in increasing myocarditis in male BALB/c mice housed in plastic cages (Fairweather 2006). In this study we found that BPS exposure had no significant effect on the expression of C3 (p=0.20), C4b (p=0.10), C3aR1 (p=0.18), C5aR1 (p=0.45), CR1/2 (p=0.47), and CR3/CD11b (p=0.08) expression by qRT-PCR (**Fig 8**). These data suggest that complement pathways are not responsible for the increase in myocarditis observed with BPS exposure.

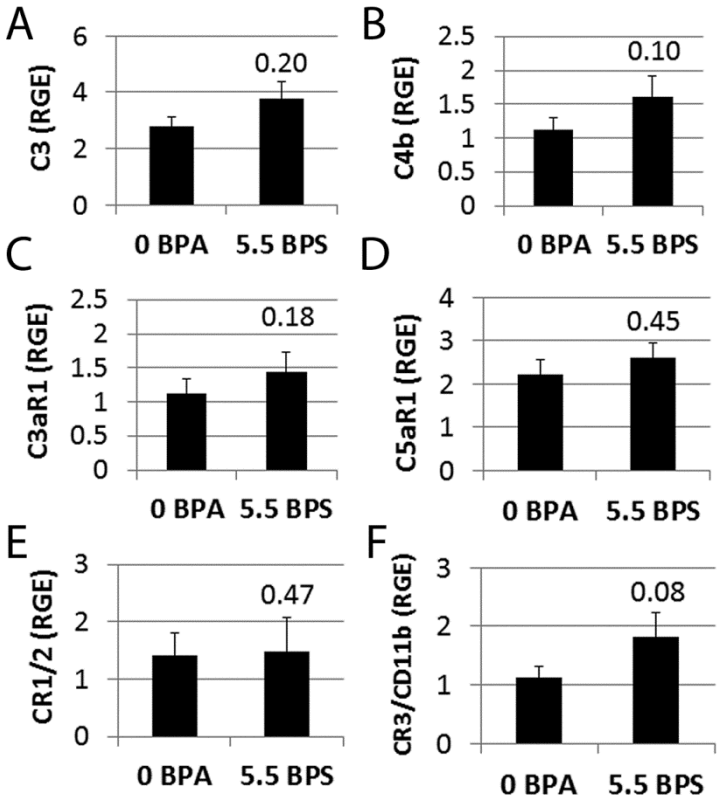

Figure 8. BPS exposure does not alter complement components in female BALB/c mice in glass cages. Female BALB/c mice were given 0 (0 BPS) or 5.5 (5.5 BPS) μg BPS /kg BW in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. Relative gene expression (RGE) of **A)** C3, **B)** C4b, **C)** C3aR1, **D)** C5aR1, **E)** CR1/2, and **F)** CR3/CD11b were compared to Hprt controls in the heart by qRT-PCR at day 10 pi comparing 0 to 5.5 μg BPS/kg BW estimated intake groups. Data show the mean +/-SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 9-10 mice/ group. 5 μg BPA/kg BW was obtained by dosing water with 25 μg BPA/L.

BPS exposure increases TLR2 and caspase-1 in the heart during myocarditis in BALB/c mice in glass cages

We showed previously that components associated with TLR4 signaling (i.e., IL-1R, caspase-1, IL-1β and IL-18) are upregulated in the heart during CVB3 myocarditis in male BALB/c mice in plastic cages (Coronado 2012, Fairweather 2015, Frisancho 2006, Frisancho 2007, Frisancho 2009, Roberts 2013). TLR2 has been found to be associated with increased myocarditis and DCM in patients (Zhang 2009). In this study we found that BPS exposure significantly increased expression of TLR2 (p=0.02) and caspase-1 (p=0.009) in the heart during acute myocarditis (**Fig 9**). IL1R2 was borderline increased (p=0.07) (**Fig 9C**). TLR4 was not significantly changed with BPS exposure compared to control water (p=0.43) (**Fig 9B**). These data suggest that BPS increases myocarditis by elevating TLR2 and caspase-1 expression in the heart.

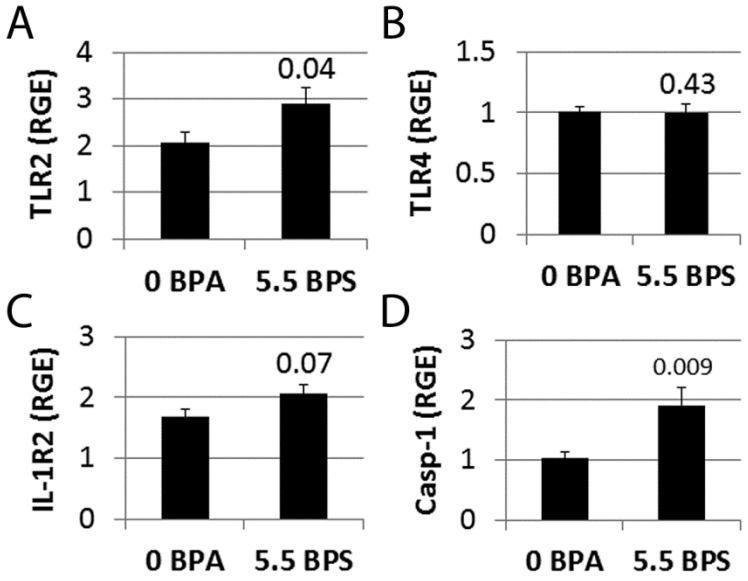

Figure 9. BPS exposure of female BALB/c mice activates TLR2 and caspase-1 during myocarditis. Female BALB/c mice were given 0 (0 BPS) or 5.5 (5.5 BPS) μg BPS /kg BW in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. Relative gene expression (RGE) of inflammasome genes vs. the housekeeping control Hprt for **A)** TLR2, **B)** TLR4, **C)** IL-1R2, and **D)** caspase-1 (Casp-1) were examined in whole

hearts by qRT-PCR at day 10 pi comparing 0 to 5.5 μg BPS/kg BW groups. Data show the mean +/- SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 9-10 mice/ group. 5.5 μg BPS/kg BW was obtained by dosing water with 27.5 μg BPS/L.

BPS exposure does not alter genes associated with remodeling and fibrosis in female BALB/c mice in glass cages

Because BPA affected genes associated with remodeling in female BALB/c mice in plastic cages (**Chapter 4**), we examined whether BPS promoted factors associated with cardiac remodeling during acute myocarditis- a timepoint when profibrotic genes/proteins are elevated but histologic fibrosis (i.e., collagen deposition) is not yet present (Coronado 2012). We found that BPS exposure did not significantly alter expression of Timp1, SerpinA3n, or TGFβ1 compared to control water by qRT-PCR (**Fig 10**).

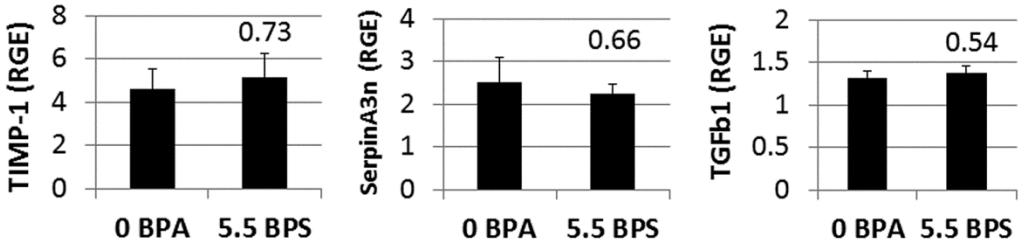

Figure 10. BPS exposure does not alter remodeling genes in the heart of female BALB/c mice during myocarditis in glass cages. Female BALB/c mice were given 0 (0 BPS) or 5.5 (5.5 BPS) μg BPS /kg BW in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0 and hearts were harvested at day 10 pi. Relative gene expression (RGE) of **A)** Timp1, **B)** Serpin A3n, and **C)** TGFβ1 vs. the housekeeping gene Hprt were analyzed in whole hearts by qRT-PCR at day 10 pi comparing 0 to 5.5 μg BPS/kg BW groups Data show the mean +/-SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 9-10 mice/ group. 5.5 μg BPS/kg BW was obtained by dosing water with 27.5 μg BPS/L.

BPS exposure to female BALB/c mice in glass cages increases ERα expression during myocarditis

In **Chapter 4** we found that BPA exposure decreased ERα and decreased ERβ in female BALB/c mice housed in plastic cages. In this Chapter, we found that BPS exposure increased the expression ERα (p=0.003) in the heart of female BALB/c mice during CVB3 myocarditis housed in glass cages, but did not significantly affect the gene expression of the other sex hormone receptors (**Fig 11**).

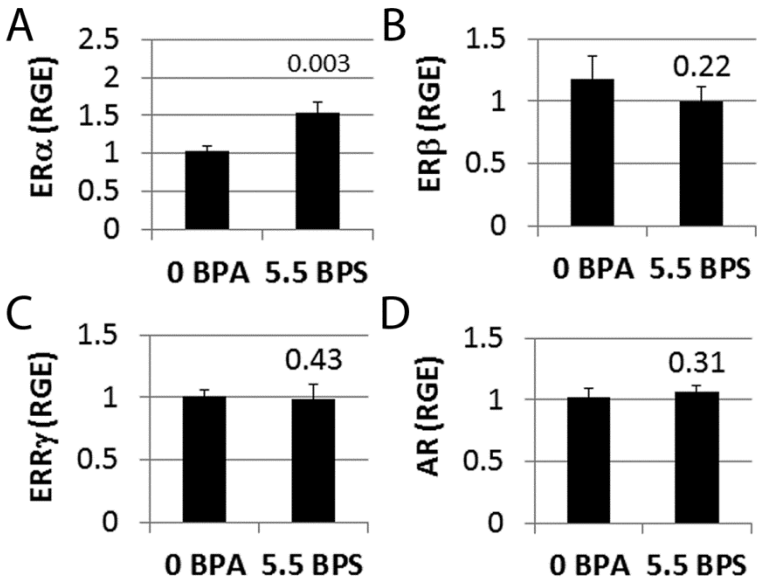

Figure 11. BPS exposure increases ERα levels in the heart of female BALB/c mice housed in glass cages. Female BALB/c mice were given 0 or 5.5 μg/kg BW bisphenol S (0 BPS vs. 5 BPS) in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0. BPS exposure continued from day 0 to 10 pi. Relative gene expression (RGE) vs. Hprt controls of **A)** ERα, **B)** ERβ, **C)** ERRγ, and **D)** AR mRNA by qRT-PCR at day 10 pi comparing 0 μg BPS/kg BW and 5.5 μg BPS/kg BW estimated intake groups. Data show the mean ±SEM using a two-tailed

Student's *t* or Mann-Whitney rank test with 9-10 mice/ group. 5.5 μg BPS/kg BW was obtained by dosing water with 27.5 μg BPS/L.

BPS exposure does not affect VDR or Vitamin D metabolism enzymes in the heart of female BALB/c mice with myocarditis housed in glass cages

In **Chapter 3** we showed that VDR deficient female mice had significantly increased acute myocarditis (**Chapter 3, Fig 3**), indicating that VDR signaling reduces CVB3 myocarditis in female mice. Furthermore, we found that VDR deficient female mice had significantly increased expression of markers for T cells (**Chapter 3, Fig 4**), IFNγ and IL-17A (**Chapter 3, Fig 5**), M1 macrophages (**Chapter 3, Fig 6**) and TLR4 activation (**Chapter 3, Fig 7**) in the heart during acute CVB3 myocarditis. These are the same findings we observed in WT female mice with CVB3 myocarditis exposed to BPA (**Results of Chapter 4**). For this reason we examined the expression of VDR and the cytochrome p450s that lead to active VitD in the heart during acute myocarditis in BPS exposed mice. We found that neither VDR nor the enzymes important in its metabolism were altered in the heart with BPS exposure (**Fig 12**). These data indicate that VDR signaling does not appear to be the mechanism whereby BPS increases myocarditis.

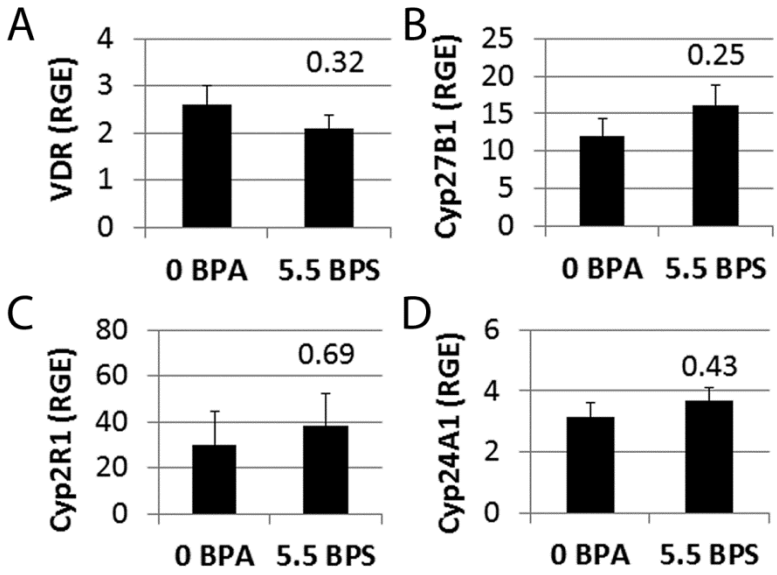

Figure 12. BPS exposure does not affect VDR or Vitamin D metabolism enzyme expression in the heart of female BALB/c mice with myocarditis housed in glass cages. Female BALB/c mice were given 0 or 5.5 μg/kg BW bisphenol S (0 BPS vs. 5 BPS) in their drinking water for 2 weeks and then injected ip with 10^3 PFU of CVB3 ip on day 0. BPS exposure continued from day 0 to 10 pi. Relative gene expression (RGE) of **A)** VDR, **B)** Cyp27B1, **C)** Cyp2R1 and **D)** Cyp24A1 vs. the housekeeping gene Hprt were analyzed in whole hearts by qRT-PCR at day 10 pi comparing 0 to 5.5 μg BPS/kg BW groups. Data show the mean +/-SEM using a two-tailed Student's *t* or Mann-Whitney rank test with 9-10 mice/ group. 5.5 μg BPS/kg BW was obtained by dosing water with 27.5 μg BPS/L.

Discussion

In this study we found that BPS exposure to adult female BALB/c mice housed in glass cages significantly increased myocarditis at day 10 pi at a high human relevant dose (5.5 μg BPS/kg BW) compared to control water. Using qRT-PCR to determine viral expression in the heart, virus was not altered by BPS exposure suggesting that increased myocarditis was not due to BPS altering viral replication. Similar to our findings with BPA, BPS exposure increased myocarditis by increasing the immune cell infiltrate containing CD4 and CD8 T cells and mast cells. In future studies we will examine mast cell numbers and degranulation using histology. ERα expression was significantly increased in the heart of female BALB/c mice during myocarditis, but the expression of other hormone receptors was unchanged according to qRT-PCR. TLR2 and caspase-1 expression was significantly increased in females after BPS exposure, similar to our findings in **Chapter 4**. Additionally, BPS was found to increase M1 proinflammatory macrophage markers, a cell type that is usually increased in male BALB/c mice. Thus, BPS exposure converted the cardioprotective female immune response to a proinflammatory immune response associated with increased myocarditis.

BPS is thought to act primarily through ERβ rather than ERα (Molina-Molina 2013, Gao 2015). We found that BPS significantly increased ERα expression. This upregulation of ERα by BPS is likely to be important for increasing T cell-mediated inflammation during myocarditis in female BALB/c mice. It may also contribute to mast cell activation. We found that BPS exposure did not significantly alter ERβ mRNA gene expression in the heart, but significantly increased ERα. This is contradictory to the literature where BPS is thought to bind to and cause gene changes mainly through ERβ. In order to further investigate the effect

of BPS on ER activation we will need to repeat these experiments and examine ER phosphorylation using Western blot and flow cytometric analysis.

With BPA being removed from plastic materials, the study of the effect of BPS on myocarditis will become increasingly important. We previously found that BPA exposure in the drinking water of female BALB/c mice housed in plastic cages increased myocarditis in a similar manner to BPS exposure. These findings were in contrast to our results for BPA exposure in glass cages, which had the opposite effect (**Chapter 6**). Because of the wide-spread use of BPS in water bottles and other food products our results suggest that understanding the immunological effects of BPS exposure are going to be critically important.

Chapter 10

General Discussion and Future Directions

General Discussion

In this Thesis we have shown that BPA exposure effects CVB3 myocarditis based on sex, strain of mouse, and type of caging. The only apparent dose response we observed for BPA was its ability to activate pericardial mast cells in female BALB/c mice housed in plastic cages. An important next step will be to examine lower doses of BPA to determine the lowest dose that can significantly alter myocarditis. We obtained opposite results for BPA exposure in female BALB/c mice housed in plastic vs. glass cages. One possible explanation for this finding is that different doses of estrogen/estradiol can have opposite effects on inflammation; with low levels of estrogen being proinflammatory while high levels are anti-inflammatory (Cutolo 1996, Accardo 1996, Hall 2001, Cutolo 2012, Jochmanova 2015, Jochmanova 2016). A similar dichotomy was observed with BPA exposures where a high dose was found to increase Th2-type immune responses (Lee 2010, Rogers 2013) and a low dose exposure found to increase Th1-type immune responses (Yoshino 2003, Yoshino 2004, Rogers 2013). Other studies found that BPA could either upregulate (Valentino 2013, Thompson 2015) or downregulate (Goto 2004, Yin 2007, Rogers 2013, Thompson 2015) proinflammatory cytokines (IL-6, IL-8, IL-17, and tumor necrosis factor-α) and immune cell proliferation depending on the study. Thus, many animal studies show conflicting information about the effect of BPA exposure on the immune response, with some studies finding that BPA upregulates Th1 responses and immune cell numbers while others find it decreases them. These differences in experimental results have been attributed to investigators using different doses, time points, and age at BPA exposure (i.e., prenatal, childhood, adult) (Kharrazian 2014). However, a major issue that is likely to confound dose

analysis that has been overlooked by most investigators is the type of cages and water bottles that are being used to conduct experiments on the role of endocrine disruptors.

Future Directions

In the future we plan to examine the effect of VitD on acute myocarditis in male and female BALB/c and BL/6 mice by feeding them a VitD deficient diet to determine if it replicates the results we found with VDR-/- mice. We also plan to examine the effect of VitD supplementation on CVB3 myocarditis in male and female BALB/c and BL/6 mice using previously defined doses of VitD added to VitD deficient chow. Our current studies used VDR knockout mice, which is an extreme model of deficiency. Therefore, using a diet deficient in VitD paired with blocking UVB exposure through cages would be necessary to determine the true effect of VitD. Additionally, many physicians are now recommending/ prescribing VitD to their patients believing that it is generally protective. However, studies that have examined VitD supplementation have generally not taken into account that VitD is a sex steroid and not examined their data according to sex (or age). It is quite possible that VitD supplementation will have a different effect than what we observed for VDR-/- mice.

We have now developed a strong "descriptive" understanding of the effect of BPA on acute CVB3 myocarditis and the next step is to prove mechanisms that have been revealed by our initial analysis. We plan to use bead separation, FACS analysis, and Western blot as techniques to further determine the mechanism of how BPA is acting in our model. In order to determine if BPA is acting on immune cells specifically or cardiomyoctyes we plan to use magnetic bead isolation of immune cells and cardiomyocytes from digested hearts exposed to varying levels of BPA during myocarditis from both sexes to determine if the changes in

important genes (i.e., ERs, VDR, CRs, and TLR4) due to BPA exposure are present on CD45+ immune cells or cardiac resident cell types (cells that did not bind to CD45+ magnetic beads). Further, we will isolate live CD45+ immune cells from digested hearts during myocarditis for flow cytometric (FACS) analysis assessing the expression of the hormone receptors on/in specific immune cell types. In our current studies we found that BPA activated/phosphorylated the ERs using Western blot. Another possible mechanism that we will explore in the future will be the effect of BPA on non-genomic signaling from membrane ERs. It has been found that BPA can affect non-genomic pathways such as the ERK/MAPK and STAT pathways, both of which can lead to activation of the immune response (Alonso-Magdalena 2005, Watson 2005, Thomas 2006, Watson 2007, Alonso-Magdalena 2008, Vandenberg 2009, Wildner 2013, Kharrazian 2014).

After we confirm the effect of BPA exposure on genomic and non-genomic signaling pathways of ERs, we will identify which ER (i.e., ERα or ERβ) or non-genomic pathway (i.e., ERK, STAT, etc.) that BPA is acting through using antagonists against the specific receptor or pathway. This will allow us to determine how BPA signals cells leading to increased (or decreased) myocarditis.

In future studies we will further assess BPA levels in water from water bottles, sera and urine using LC-MS/MS. We will also attempt to get a better understanding of the chemicals that are being released from the plastic cages and water bottles by sending samples collected from water left sitting in cages or water bottles to collaborators who will use mass spectrometry to determine the chemical composition. Once we determine what chemicals are leaching from the plastic cages and water bottles that are affecting our experiments, we

would like to design further exposure experiments to better understand their effect on sex differences in CVB3 myocarditis.

There are several possible mechanisms for how BPA could alter myocardial inflammation that have not been the focus of this thesis. For example, studies by other investigators have found that BPA can directly bind to the arylhydrocarbon receptor and the thyroid hormone receptor (Kharrazian 2014,Vandenberg 2009), peroxisome proliferator-activated receptors (Kharrazian 2014) and the G-protein coupled receptor for estrogen (GPR30) which has been found to be a nuclear ER (Acconcia 2015). Another possible mechanism for the action of BPA on myocarditis that could be increasing disease is through epigenetic modification. Studies have found that BPA increases cardiomyopathy and decreases cardiac function by DNA methylation (Jiang 2014). Studies looking at other disease endpoints have also found that BPA can affect DNA methylation (Cabaton 2013, Kundakovic 2013, O'Brien 2014, Jochmanova 2015) and cause DNA epigenetic reprograming (André 2009, Wildner 2013, Kharrazian 2014). It is known that epigenetics can regulate the immune response through shifting the Th1/Th2 response and thereby alter cytokine levels (Hirahara 2011). Both of these processes are involved in myocarditis disease pathogenesis and could be important in driving disease by potentially effecting immune cells (Ivashkiv 2016). BPA could also increase inflammation by elevating oxidative stress (Tiwari 2012, Rezg 2014). These are just a few alternative mechanisms that could explain how BPA exposure alters inflammation leading to increased (or decreased) myocarditis in our mouse model.

BPA has been found in some studies to have sex-specific effects on CVDs. For example, BPA was found to increase cardiac remodeling only in males, but in females BPA

affected cardiac function (Melzer 2010, Gao 2014). Others have found that BPA exposure increases cardiac remodeling, decreases cardiac function, activates TGFβ, and increases cardiac fibroblasts through the ERK1/2 pathway (Hu 2016). We found that BPA exposure upregulated genes associated with remodeling and fibrosis, suggesting that BPA exposure could increase the risk of progression to chronic myocarditis and DCM. To determine if BPA exposure alters the progression to DCM in males or females we will use the same exposure method as described for acute myocarditis and harvest at day 35 pi (rather than day 10 pi) with continued BPA exposure from day 0 to day 35 pi. At day 35 pi we will perform echocardiograms to assess cardiac function for mice exposed to BPA compared to control water exposure. We will use histological staining to quantify chronic myocarditis by H&E and remodeling using trichrome blue.

Understanding whether prenatal exposure to BPA and/or other EDs increases adult myocarditis and DCM could have a large impact on our understanding of the pathogenesis of disease. Our finding that BPA exposure activated pericardial mast cells in the heart could be one possible reason why women in their 3rd trimester of pregnancy sometimes develop myocarditis and heart failure (called peripartum cardiomyopathy/ PPCM) (Blauwet 2011). The reason that PPCM develops is not well understood. Therefore, determining the effect of BPA on prenatal exposure in an animal model could help elucidate one possible cause and mechanism for a disease, PPCM, which does not have a good animal model.

There are currently no human studies looking at the association of BPA levels with myocarditis, DCM or PPCM. Our laboratory is a member of the Peri/Myocarditis, DCM and PPCM Biobank at Mayo Clinic where we are collecting sera, peripheral blood mononuclear cells, and myocarditis biopsy tissues from patients to study mechanisms for sex differences in

disease comparing our mouse model to clinical samples. We are going to begin collecting urine as well in order to assess BPA levels in patients with myocarditis and DCM.

We have only just begun our analysis of the effect of BPS on CVB3 myocarditis. These studies will continue. BPF is another plastic being used to replace BPA in plastics. Meta-studies have found that BPF may be even more potent than BPA using estrogenic assays (Molina-Molina 2013, Rochester 2015). Other EDs that could increase the risk for myocarditis and DCM and could be studied in our model include genistein, which is in soy products, dioxins, pesticides, and arsenic, which is known to increase CVD, for example (Dietert 2012).

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Zhu, J., Yamane, H., & Paul, W. E. (2010). Differentiation of effector CD4 T cell populations (*). *Annual Review of Immunology*, *28*, 445–89.

Zittermann, A., & Gummert, J. F. (2010). Sun, vitamin D, and cardiovascular disease. *Journal of Photochemistry and Photobiology. B, Biology*, *101*(2), 124–9.

Zoeller, R. T., & Vandenberg, L. N. (2015). Assessing dose-response relationships for endocrine disrupting chemicals (EDCs): a focus on non-monotonicity. *Environmental Health : A Global Access Science Source*, *14*, 42.

Zold E, Barta Z, B. E. (2011). Vitamin D deficiency and connective tissue disease. In *Vitamins and Hormones: Vitamins and the Immune System* (pp. 261–286). Amsterdam: (G Latwick, Ed), Elsevier.

Curriculum Vitae

Katelyn Ann Bruno
Bruno.katelyn@mayo.edu
Birth date and location: May 18th, 1989 Springfield, MO

Education:

BS, Criminal Justice, Sam Houston State University (9/2007-8/2010)
Concentration: Forensic Science

BS, Professional Chemistry ACS Certified, Sam Houston State University (9/2007-5/2011)
Concentrations: Toxicology, Analytical Chemistry, Forensic Science

Thesis: Analytical Method Development for Determining the Biomarker, 2-Aminothiazoline-4-Carboxylic Acid (ATCA), in Mice Liver after Cyanide Exposure
PhD, Environmental Health Sciences, Johns Hopkins University (7/2011-8/2016)
PhD Track in Molecular & Translational Toxicology
Concentrations: Cardiovascular Disease, Immunology, Toxicology, Sex Differences

Dissertation: Effect of Environmentally-derived Sex Steroids on Coxsackievirus B3 Myocarditis: Focus on Vitamin D and Bisphenol A

Advisor: DeLisa Fairweather, PhD Johns Hopkins University

Experience:

(8/2009-12/2009) Teaching Assistant and Tutor, Sam Houston State University (Course: General Chemistry I)

(1/20010-5/2011) Teaching Assistant and Tutor, Sam Houston State University (Course: Organic Chemistry I)

(5/2009-5/2011) Student Research Assistant in Ilona Petrikovics Laboratory, Sam Houston State University

(8/2013-10/2013) Teaching Assistant, Johns Hopkins University (Course: Principles of Environmental Health I)

(10/2013-12/2013) Teaching Assistant, Johns Hopkins University (Course: Principles of Environmental Health II)

(5/2010-5/2011) Forensic Chemist Intern, DEA South Central Laboratory, Dallas, TX

(3/2013-5/2013) Teaching Assistant, Johns Hopkins University (Course: Immunology of Environmental Diseases)

(1/2014-3/2014) Teaching Assistant, Johns Hopkins University (Course: Environmental and Occupational Health Law and Policy)

(3/2014-5/2014) Teaching Assistant, Johns Hopkins University (Course: Introduction to Environmental and Occupational Health Law)

Research Skills:

Training in microarray analysis

Training in molecular biology techniques including RT-PCR

Training in minor surgical techniques including gonadectomy of male and female mice

Genotyping using gel electrophoresis

Basics in Flow Cytometry/FACS

Cell culture

Plaque Assay

Histological Analysis of tissue sections

Animal Husbandry (maintain mouse colonies)

Honorary Positions:

(9/2011-8/2016) Johns Hopkins Bloomberg School of Public Health (JHSPH) Diversity Student Ambassador

(9/2013-9/2014) Environmental Health Student Group (EHSSO), Student Assembly Representative

(9/2014-12/2014) Environmental Health Student Group (EHSSO), Treasurer

(6/2014-6/2015) Johns Hopkins Bloomberg School of Public Health (JHSPH) Student Assembly, Vice President of Communications

(9/2013-current) Myocarditis Foundation, Director of Communications

(6/2016-current) Myocarditis Foundation, Assistant Secretary of the Board

Honors & Awards:

(2007 - 2011) Sam Houston State University (SHSU), President's and Dean's List

(2008) Most Outstanding First-Year Student Leader Sammy Award

(2010) Society of Toxicology Undergraduate Education Program Travel Grant

(2010) Won 1st place poster presentation at the Sam Houston State University College of Criminal Justice 2nd Annual Undergraduate Conference.

(2010) American Academy of Forensic Science, Forensic Sciences Foundation, Emerging Forensic Scientist Award Paper Presenter

(2011) Boe Paper Award Representative for SHSU in *Great Plains Honors Council Conference*

(2011) Academic Distinction in Professional Chemistry

(2013) 1st place poster presentation at *What a Difference an X Makes Conference* hosted by Society of Women's Health Research in Washington D.C. July 18-19. Presentation Title: "Vitamin D receptor signaling reduces inflammation in female mice with myocarditis, but increases inflammation in males: implications for translation studies."

(2014) Poster award for American Association of Immunologist meeting in Pittsburg, PA April 4-7. Presentation Title: "Bisphenol A (BPA) exacerbates acute myocarditis in female BALB/c by activating mast cells and the inflammasome"

Professional Memberships:

(2009 - current) Golden Key International Honor Society (top 15% of university students internationally)

(2009 - current) Alpha Chi Honors Society (top 10% of university students in the US)

(2009 - current) Society of Toxicology, Women in Toxicology and Immunotoxicology Subsection

(2012 - current) Organization for the Study of Sex Differences (OSSD)

(2012 - current) The Endocrine Society

(2012 - current) American Association of Immunologists (AAI)

(2014 - current) Heart Failure Society of America (HFSA)

Publications:

Yu JCC, Martin S, Nasr J, Stafford KA, Thompson DE, Petrikovics I (2012) LC-MS/MS analysis of 2-aminothiazoline-4-carboxylic acid as a forensic biomarker for cyanide poisoning. *World J Methodol*. 2: 33-41

Fairweather D, Stafford KA, Sung YK (2012) Update on coxsackievirus B3 myocarditis. *Curr Opin Rheumatol*. 24: 401-407

Presentations:

Stafford K, Jackson, R, Yu J, Petrikovics I, 2009, Analytical Method Development for Determining the Biomarker, 2-Aminothiazoline-4-Carboxylic Acid(ATCA), in Mice Liver After Cyanide Exposure. Poster presentation at 65th Southeast Regional Meeting of the American Chemical Society. El Paso, TX.

Martin S, Kuzmicheva G, Stafford K, Petrikovics I, 2009, Determining the Optimal Condition for Rhodanese Incorporation into Liposomes. Poster presentation at 65th Southeast Regional Meeting of the American Chemical Society. El Paso, TX.

Stafford K, Jackson R, Simons K, Yu J, Petrikovics I, 2010, Analytical Method Development for Determining the Biomarker, 2-Aminothiazoline-4-Carboxylic Acid (ATCA), in Mice Liver After Cyanide Exposure. Poster presentation at 62nd Annual Meeting of the American Academy of Forensic Science. Seattle, WA.

Stafford K, Moss K, Lindgren N, Bytheway J, 2010, Post Mortem Interval of Surface Remains during Spring in Southeast Texas. Poster presentation at 62nd Annual Meeting of the American Academy of Forensic Science. Seattle, WA.

Stafford K, Yu J, Myagmarjaya B, Petrikovics I, 2010, Sample Preparation Method Development for Determining the Biomarker, 2-Aminothiazoline-4-Carboxylic Acid (ATCA), from Mice Liver after Cyanide Exposure. Poster presentation at 113th Annual Meeting of the Texas Academy of Science. Stephenville, TX.

Yu J, Petrikovics I, Jackson R, Stafford K, 2010, Analytical Method Development for Determining the Biomarker Cyanide Metabolite, 2-Aminothiazoline-4-Carboxylic Acid, in Mice Liver After Cyanide Exposure. Poster presentation at 49th Annual Meeting of the Society of Toxicology. Salt Lake City, UT.

Stafford K, Yu J, Petrikovics I, 2010, Development of a New Analytical Method for the Determination of the Biomarker, 2-Aminothiazoline-4-Carboxylic Acid (ATCA), in Mice after Cyanide Exposure. Poster presentation at 2nd Annual Sam Houston State University College of Criminal Justice Undergraduate Conference. Huntsville, TX.

Stafford K, 2010, New Analytical Method Development for Determination of the Biomarker, 2-Aminothiazoline-4-Carboxylic Acid (ATCA), in Mice after Cyanide Exposure. Oral Presentation at Pin Oak Middle School Visiting Scientist Day. Houston, TX.

Stafford K, Yu J, Petrikovics I, 2010, Development of a New Analytical Method for the Determination of the Biomarker, 2-Aminothiazoline-4-Carboxylic Acid (ATCA), in Mice after Cyanide Exposure. Poster presentation at 3rd Annual Sam Houston State University Honors Undergraduate Research Symposium. Huntsville, TX.

Stafford K, Moss K, Lindgren N, Bytheway J, 2010, Post Mortem Interval of Surface Remains during Spring in Southeast Texas. Poster presentation at 3rd Annual Sam Houston State University Honors Undergraduate Research Symposium. Huntsville, TX.

Petrikovics I, Stafford K, Thompson D, Jayanna P, Yu J, 2010, Determining the Biomarker Cyanide Metabolite 2-Aminothiazoline-4-Carboxylic Acid in Mice Liver after Cyanide Exposure. Poster presentation at Bioscience Meeting. Baltimore, MD.

Stafford K, Glanville K, 2010, d,l-Methamphetamine Synthesis via Phenyl-2-Propanone from Phenyl-2-Propanol. Oral presentation at DEA Synthesis Lecture Series. Dallas, TX.

Petrikovics I, Stafford K, Thompson D, Jayanna P, Yu J, 2010, Determining the Biomarker Cyanide Metabolite 2-Aminothiazoline-4-Carboxylic Acid in Mice Liver after Cyanide Exposure. Poster presentation at XII. International Congress of Toxicology. Barcelona, Spain.

Stafford K, Glanville K, 2010, U.S. Department of Justice: Drug Enforcement Administration South Central Laboratory. Oral Presentation at Corinth Police Department Summer Camp. Corinth, TX.

Stafford K, Yu J, Petrikovics I, 2010, Analytical Method Development for the Quantization of Cyanide Exposure Biomarker, ATCA, in Mice Sample. Poster presentation at Southwest Association of Forensic Science Meeting. Dallas, TX.

Stafford K, Yu J, Petrikovics I, 2011, Determining the biomarker cyanide metabolite 2-aminothiazoline-4-carboxylic acid in mice liver after cyanide exposure. Oral presentation at Great Plains Honors Council Conference. Fort Worth, TX.

Stafford K, Cooper L, Douglass E, Brandt J, Bucek A, Coronado M, Kew R, Fairweather D, 2012, Sex differences in the effect of vitamin D on inflammatory heart disease: protective in women but damaging in men, Poster presentation at Organization for the Study of Sex Differences. Baltimore, MD.

Stafford K, 2012, Sex differences in the effect of Vitamin D on inflammatory heart disease: protective in women but damaging in men. Oral presentation at Advancing Excellence in Gender, Sex and Health Research Conference. Montreal Canada.

Stafford K, Cooper L, Douglass E, Brandt J, Bucek A, Coronado M, Kew R, Fairweather D, 2013, Vitamin D receptor signaling reduces inflammation in female mice with myocarditis, but increases inflammation in males: implications for translation studies, Poster presentation at Organization for the Study of Sex Differences. Weehawken, NY.

Stafford K, Molina F, Clifford M, Douglass E, Cooper L, Brandt J, Bucek A, Fairweather D, 2013, Vitamin D receptor signaling reduces inflammation in female mice with myocarditis, but increases inflammation in males: implications for translation studies, Poster presentation at Society for Women's Health Research. Washington DC.

Stafford K, Cooper L, Douglass E, Brandt J, Bucek A, Coronado M, Kew R, Fairweather D, 2013, Sex differences in the effect of vitamin D on inflammatory heart disease: protective in women but damaging in men, Poster presentation at American Association of Immunologists. Honolulu, Hawaii.

Stafford K, Douglass E, Cooper L, Bucek A, Coronado M, Brandt J, Fairweather D, 2013, Vitamin D receptor signaling reduces inflammation in female mice with myocarditis, but increases inflammation in males: implications for translation studies. Poster presentation at Heart Faliure Society of America. Orlando, FL.

Stafford K, Greyner H, Bucek A, Fairweather D, 2013, Bisphenol A (BPA) increases acute autoimmune myocarditis in female BALB/c mice through ERb, the inflammasome and mast cells. Poster presentation at Heart Faliure Society of America. Orlando, FL.

Stafford K, Greyner H, Molina F, Bucek A, Fairweather D, 2014, Bisphenol A (BPA) exacerbates acute myocarditis in female BALB/c by activating mast cells and the inflammasome, Poster presentation at American Association of Immunologist. Pittsburg, PA.

Stafford K, Greyner H, Molina F, Bucek A, Fairweather D, 2014, Bisphenol A (BPA) exacerbates acute myocarditis in female BALB/c by activating mast cells and the inflammasome, Poster presentation at Organization for the Study of Sex Differences. Minneapolis, MN.

Bruno K, Greyner H, Yang A, Mathews J, Greyner H, Molina F, Bucek A, Fairweather D, 2016, Bisphenol A (BPA) and Bisphenol S (BPS) increase Coxsackievirus B3 myocarditis in female and male BALB/c mice by activating mast cells, increasing immune cell infiltrate and activating the inflammasome, Poster presentation at American Association of Immunologist. Seattle, WA.

Grants and Research Support:

(2009-2010) Department of Defense (DoD) Student Research Grant for undergraduate research, Sam Houston State University, Huntsville, TX

(2009-2011) Burroughs's Physics and Chemistry Scholarship, Sam Houston State University, Huntsville, TX

(2011-Current) National Institute of Environmental Health Sciences (NIEHS) Training Grant (ES07141), PhD student project: “Sex hormones and endocrine disruptor’s influence on acute myocarditis”

(2013-Current) National Institute of Environmental Health Sciences (NIEHS) RFA-ES-13-011 “The role of environmental exposures in the development of autoimmune disease”, R21